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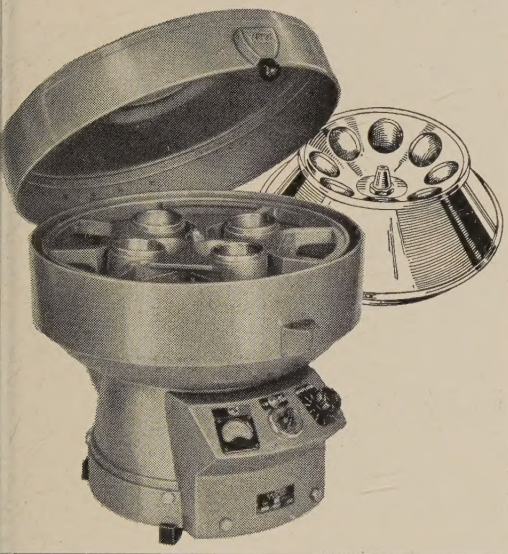

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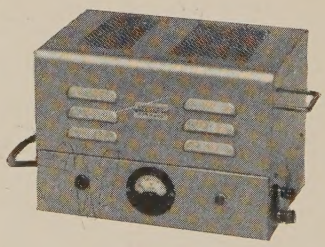

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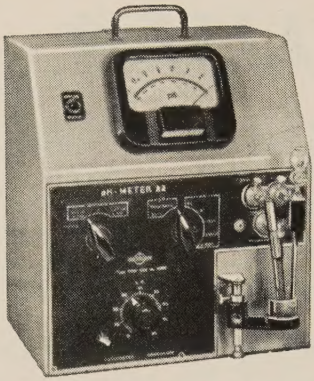

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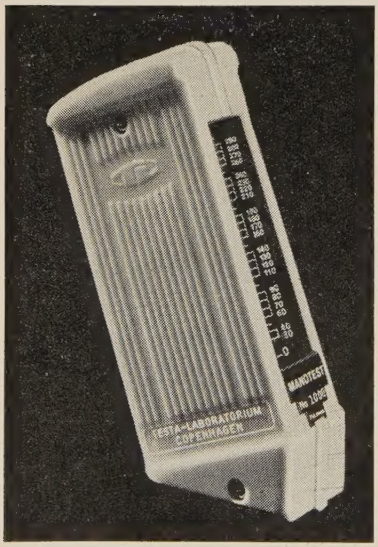

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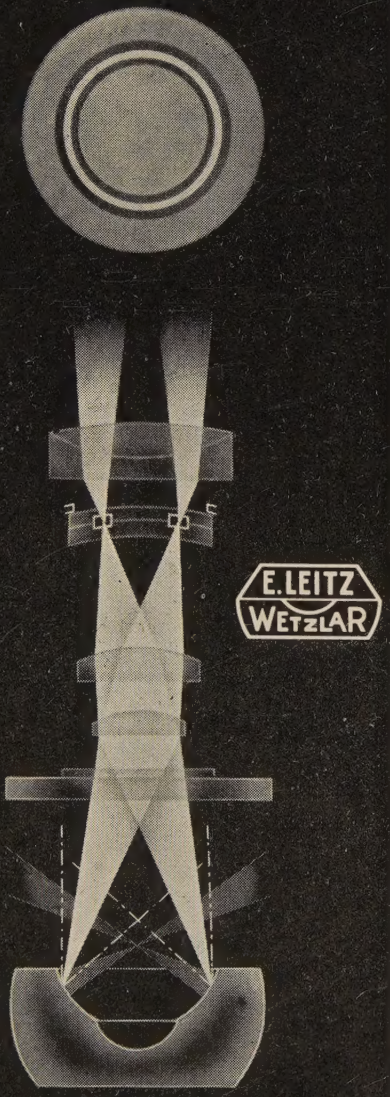

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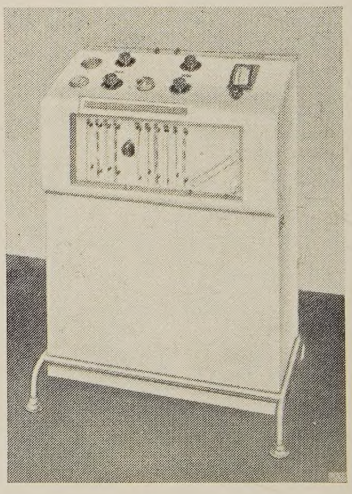

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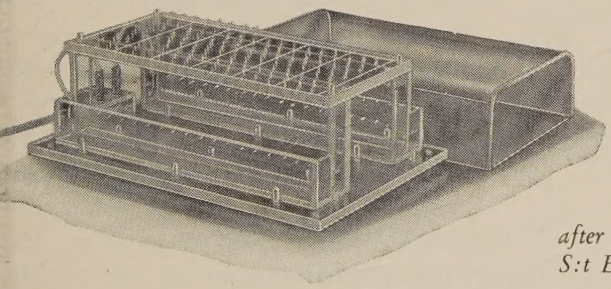

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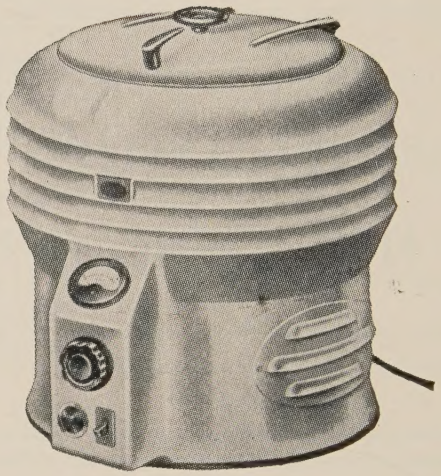

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ON THE IODIDE-OXIDISING SUBSTANCE IN THE URINE

I. CLINICAL STUDIES

N. HALLMAN AND E. KULONEN

From the Children's Clinic and from the Department of Medical Chemistry, University of Helsinki, Finland

(Received for publication Febr. 2, 1954)

Fishberg (1948) has demonstrated that in ne disease conditions associated with de- ency in ascorbic acid, substances are nd in the urine which produce *in vitro* formation of methemoglobin. This for- tion of methemoglobin in the urine, on other hand, was first established in the eedingly rare disease "autotoxic entero- ous cyanosis". For identification and ermination of the oxidising substance, hberg (1948) applied a method formerly eloped for the soluble *p*-quinones by leur, which she also adapted for quantita- e determination. The method is based on dation of iodides.

Since the problem of occurrence of these dising agents in the urine has been dealt h in the literature to an insignificant ent, we have felt justified in performing umber of studies on the urine of children fering from various diseases. The speci- ty of the reaction employed is discussed ewhere (Kulonen & Hallman, 1954).

METHOD

10 cc of 10 per cent KI and 10 cc of 2 *N* H_2SO_4 were added to 20 cc of urine. The solution turns yellowish brown if quinone is present. This was titrated with 0.01 *N* sodium thiosulphate and just before the end-point a few drops of starch solution were added as indicator. The end-point, the disappearance of the blue color, is sharp. All results are expressed in ml. of 0.01 N sodium thiosulphate used in titration.

RESULTS

The results are summarised in Table I.

The controls consisted of 272 school children, their age ranging from 8 to 11 years. Bottles for the collection of urine were distributed to them in the evening. The samples were collected next morning in school and analysed in the course of the forenoon. Simultaneously with determining the oxidising capasity of urine, qualitative studies were made of sugar and occasionally albumin in the urine. Only one case had sugar in the urine. The child was a diabetic.

Table I. *Iodide-oxidising capacity of urine in different conditions expressed in per cent of all determinations.*

	Number of cases	Number of analyses	ml 0.01 *N* Sodium thiosulphate						Reaction positive %
			0	≦ 0.2	> 0.2 ≦ 1.0	> 1.0 ≦ 5.0	> 5.0 ≦ 10.0	>10.0	
Control material	272	272	97.8	0.7	0.4	0.7	0.4	0	2.2
Prematures	22	29	89.7	3.4	6.8	0	0	0	10.3
Miscellaneous diseases	315	406	88.2	1.0	4.7	3.7	1.0	1.5	11.8
Nephrosis and nephritis	11	70	65.7	0	1.4	14.3	12.9	5.7	34.3
Diabetes mellitus (children)	101	1395	58.6	2.3	6.5	20.5	8.5	3.6	41.4
Diabetes mellitus (adults)	130	130	77.7	6.2	6.2	10.0	0	0	22.3
Total	851	2302	—	—	—	—	—	—	—

As shown by Table I, a positive response was obtained in 2.2 per cent or in 6 cases. These children also were completely healthy and had not previously had any noteworthy diseases. Renewed examinations which are not included in the table showed a negative result in 4 cases. In two cases, on the other hand, the consumption of thiosulphate was still relatively high (case I: 4.6; 6.7; 10.9; ml; case II: 5.0; 7.5; 7.6 ml).

In hospital the urine samples examined were taken directly from the routine laboratory, to which they had been taken for other investigations. The samples were collected mainly in connection with the morning work and the determinations were again performed in the course of the morning. They can in this respect be compared to the control material.

In the group of miscellaneous diseases the positive responses were clearly more numerous than in the controls. Premature infants were separated from this group, since it had been previously found that phenylalanine and tyrosine derivatives are excreted into their urine at a certain stage, and they in their turn may cause the formation of methemoglobin *in vitro*. The 22 premature infants examined had an age range of 2 to 42 days, their birthweight varied from 1270 to 2000 g and their weight in examination between 1370 and 2600 g. Three of the samples were positive, all of them, however, only to a relatively slight extent. At the time of examination all these three infants were over 25 days old and their weight exceeded 1700 g. In addition, they all received daily doses of ascorbic acid. It is noteworthy that the urine of younger prematures with a lower weight never once gave a positive result. In the whole premature group the number of positive responses just fell short of those in the group of miscellaneous diseases.

Table II. *Diabetic children at hospital. Urine samples collected during different hours.*

Urine stored hours	ml 0.01 *N* Sodium thiosulphate				Reaction positive %
	0	≦1.0	>1.0≦5.0	> 5.0	
0—6	129	15	22	2	
o'clock	76.8 %	8.9 %	13.7 %	0.6 %	23.2
0—6	117	11	25	6	
o'clock	73.6 %	6.9 %	15.7 %	3.8 %	26.4
7—12	127	14	20	14	
o'clock	72.6 %	8.0 %	11.4 %	8.0 %	27.4
13—18	89	7	26	42	
o'clock	54.3 %	5.0 %	15.5 %	25.2 %	45.7

The group of miscellaneous diseases con-ned 30 newborn infants who had been mitted on account of various disorders. this group there were 3 positive responses, th a degree approximately similar to those tained in the premature group. The diag-sis and age of those belonging to other oups was highly variable. Among the posi-e responses mention should be made of o suffering from rheumatic fever, one case lupus erythematodes, several tonsillites, stroenterites and infants who had been erated for various reasons. It was gener-y a question of fever, either present at the ment or just improved, or of a post-erative condition as already mentioned. owever, no common demarcation line can drawn between the positive and negative sponses.

Since it was seen that positive reactions re relatively numerous among those suf-ing from renal disorders and also among betics, these groups were subjected to re detailed examinations.

It must be said in the first place that the nature of the renal disorder involved does not, on the basis of our cases, greatly affect the matter under discussion. Disorders of both the nephrotic and the nephritic type produced a more or less similar number of positive responses. Every single case on whom several determinations were performed gave sometimes positive and sometimes negative reactions. The results did not seem to have any relation either to the protein content or to the sediment of the urine. Patient M.C., 11 years, can be mentioned as a typical example.

Albumin per cent: 1.0; 0; 3.8; 6.0; 8.5; 8.0; 0; 3.8; 6.0; 8.0; 7.0; 5.0.
0.01 *N* $Na_2S_2O_3$ ml: 0; 0; 0; 0; 0; 7.5; 5.3; 6.2; 1.4; 0; 0; 0.

When assessing the results it should be taken into account that for those suffering from renal diseases the whole amount of urine passed in 24 hours was collected. Consequently, part of the urine analysed was about 24 hours old.

The largest group consisted of patients suffering from diabetes. Positive reactions were elicited in 41.4 per cent, *i. e.* more than in any other group examined (Table I). It should be noted that each patient was subjected to more than one examination.

There were 25 children at the hospital and their urine was collected in four parts at 2, 8, 14 and 20 o'clock. The determinations were generally performed in the morning and in the afternoon. The samples collected at 8 o'clock and 14 o'clock had been standing for the shortest time, and the others correspondingly longer. Table II shows that the number of positive reactions was smallest

Table III. *Diabetic children examined at outpatient department. Effect of storage.*

Stored hours	Negative samples	Positive samples	Positive %
0—12	44	29	37.7
13—24	38	37	48.1
25—36	34	41	53.3
37—48	44	35	45.5

in the groups in which the sample was fresh. Strongly positive responses were clearly least in these groups. Positive as well as strongly positive responses are clearly most numerous in samples collected in the evening.

The number of children examined as outpatients was 76. Each of them had with him 4 samples taken from the urine of the two foregoing 24 hours, the output during the day separated from that at night. The responses of those patients are illustrated in Table III. If a comparison to the resident patients is made, we note that positive responses are more numerous in all groups. The number of positive samples does not increase constantly as the time of storage becomes longer.

To complement the foregoing results, examinations were also performed on some adult diabetics (Table I). This group also had fairly numerous positive reactions, *i. e.* 23.7 per cent. However, the number of strongly positive responses was clearly less than in diabetic children.

When comparing blood and urine sugars in diabetics to the foregoing results we failed to establish any correlation whatever. Determinations of blood sugar had been performed in relatively few cases. Sugar in the urine, on the other hand, had been determined in each individual sample.

Table IV. *Urine acetone in diabetic children compared with iodide-oxidising reaction.*

Acetone bodies	Oxidation of iodide		Total
	+	—	
—	367	552	919
+	63	177	240
Total	430	729	

The χ^2-test shows that the trend to the absence of acetone bodies in the iodide-oxidising urine and *vice versa* is statistically significant. ($P<.01$.)

The results were also compared with the presence of acetone bodies with qualitative determinations performed parallel with thiosulphate titration. The results varied so that positive acetone test could associate with negative as with a positive iodide-oxidising test. If, however, we attempt to coordinate all simultaneous examinations (Table IV) we se that positive acetone substances in the urine are statistically more often correlated with negative iodide oxidation than with a positive oxidising reaction ($P<.01$). This proves that the presence of acetone in the urine is evidently not the cause of positivity of the iodide-oxidising reaction but the reverse correlation is noteworthy.

COMMENT

According to earlier investigators (Fishberg, 1944), oxidation of iodide in the urine occurs if less ascorbic acid than usual is excreted into the urine. In rheumatic infections (Sealock & Silberstein, 1940) and in premature infants certain (Levine & Gordon, 1941) keto- and hydroxy-derivates of phenylalanine and tyrosine are found in

ie urine and their excretion is stopped if scorbic acid is given. Fishberg (1948) iden- fied the iodide-oxidising substance as benzo- uinone-acetic acid that might be an obli- ate intermediate in the normal catabolism tyrosine and phenylalanine.

Our results evidence that oxidation of odides by the urine *in vitro* can sometimes ccur in the samples of perfectly healthy ildren, and is frequent in the urine of ildren suffering from diabetes or different nal disorders. The ascorbic acid was not etermined in the urine of the patients of ir series, but in no case was there any inical evidence of ascorbic acid deficiency. or is, to our knowledge, this deficiency in ny particular way associated with diabetes r kidney diseases. It is also noteworthy at the presence of acetonic substances in ne urine does not increase the positivity of eaction, at any rate in diabetics.

The fact that the iodide-oxidising effect the urine is not of constant extent even the same individual, showing at times a rong reaction and disappearing again even ithin the course of the same 24 hours, sug- ests a labile disorder, which probably does not possess any marked immediate clinical significance. The increasing number of positive reactions on storage of urine also points to the possibility that we are determining a different substance than the earlier investigators.

SUMMARY

The iodide-oxidising capacity of the urine *in vitro* was studied in healthy children and in different diseases. In certain disorders the oxidising capacity is markedly more frequent than in health. Oxidation is particularly common in patients suffering from renal lesions and in diabetics (both in children and adults).

One and the same patient can have both positive and negative samples. The presence of sugar in the urine of diabetics was not seen to have correlation with the incidence of the oxidation phenomenon. The oxidising capacity tends to associate with the absence of the acetone bodies in the urines of diabetic patients.

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SPECTROPHOTOMETRIC DETERMINATION OF HEMOGLOBIN OXYGEN SATURATION

THE METHOD OF DRABKIN & SCHMIDT AS MODIFIED FOR ITS USE IN CLINICAL ROUTINE ANALYSIS

By L. KLUNGSØYR and K. FR. STØA,

WITH TECHNICAL ASSISTANCE OF SOLVEIG SØRENSEN

From the Biochemical Laboratory, University Clinic of Bergen, Norway

(Received for publication May 24, 1954)

Due to its application for diagnostic purposes in heart and lung diseases, the determination of the oxygen content in blood is of the greatest importance.

The method of Van Slyke & Neill (1924) is generally recognized as the most reliable of the methods available for determination of blood-gases. This method has been used in this laboratory for several years with satisfactory results (Rasmussen & Storstein, 1951, Storstein, 1952).

When many analyses must be run, the manometric method is time-consuming and tiring for the operator. The method includes several steps which must be carried out with the greatest care. It is therefore not surprising that, according to our experience, errors occasionally occur during routine work.

The average value for arterial hemoglobin oxygen saturation is approximately 95 per cent as determined manometrically. This value is probably 2—3 per cent too low (Drabkin & Schmidt, 1945, Roughton, Darling & Root, 1944).

It has been the purpose of this work to find a method suitable for rapid and accurate determination of oxygen saturation in blood. The method of Drabkin & Schmidt (1945) seemed promising, as it is simple and accurate. The method as modified by us is also rapid, and convenient for routine use.

PRINCIPLES OF METHOD

The spectrophotometric determination of hemoglobin oxygen saturation depends upon the differences in light absorbtion between hemoglobin (Hb) and oxyhemoglobin (HbO_2). (Fig. 1.)

Thus, for a given wavelength:

$$E_x = X\ E_{Hb} + (1 \div X)\ E_{HbO_2} \quad (1)$$

where E_{Hb}, E_{HbO_2} and E_x are the extinction coefficients of Hb, HbO_2 and the unknown mixture of the two respectively, and X is the part of the two respectively, and X is the part of the total hemoglobin present as Hb in the sample.

According to Drabkin & Schmidt, X may be determined by measuring at the actual wavelength the optical density of the anaerobically treated sample, convert the Hb and HbO_2 into some common from and then determine the total hemoglobin present in the sample. From empirically determined values for E_{Hb} and E_{HbO_2}, the optical densities of pure Hb and of pure HbO_2 may be calculated for the actual light path and the hemoglobin concentration of the sample. The optical densities for pure Hb and HbO_2

lutions may also be determined directly treating the sample with sodium dithionite, and by saturating it with oxygen. X may en be determined according to equation Drabkin & Schmidt determined X simultaneously at 4 different wavelengths, whereby ey obtained a high degree of accuracy.

The quotients between the optical densities at two suitable wavelengths are constants, characteristic for pure Hb and for ure HbO_2, and independent of light path d hemoglobin concentration (Lambrechts, Lefevre & Cornil, 1942; Zijlstra, 1953). A quotient determined in this way in a mixture of Hb and HbO_2, will have an intermediate value between the quotients of the pure substances. Thus, it is possible to calculate the relative content of Hb and HbO_2 in a sample by measuring the optical densities in the sample at two wavelengths. 558 and 575 millimicrons are suitable, as at these wavelengths the difference between the quotients for pure Hb and HbO_2 is great. (See Fig. 1.)

We set:

$$\frac{D^{575}_{HbO_2}}{D^{558}_{HbO_2}} = q_1; \quad \frac{D^{575}_{Hb}}{D^{558}_{Hb}} = q_2; \quad \frac{D^{575}_{Hb}}{D^{575}_{HbO_2}} = r; \quad \frac{D^{575}_{x}}{D^{558}_{x}} = q_x.$$

Taking $D^{558}_{HbO_2}$ for unity, we get:

$$D^{558}_{HbO_2} = 1; \quad D^{575}_{HbO_2} = q_1; \quad D^{575}_{Hb} = q_1 r; \quad D^{558}_{Hb} = q_1 r/q_2.$$

From equation 1 we get:

$$\frac{X D^{575}_{Hb} + (1-X) D^{575}_{HbO_2}}{X D^{558}_{Hb} + (1-X) D^{558}_{HbO_2}} = q_x$$

(2) or: $$\frac{(q_1 r X + q_1 (1-X)}{(q_1 r/q_2) X + 1 \div X} = q_x; \quad \frac{q_1 \div q_x}{q_x (q_1 r/q_2 - 1) + q_1 - q_1 r} = X.$$

In this equation q_x is measured and the others are constants that may be determined once and for all.

DESIGN OF CUVETTES

For measuring the optical densities in hemoed blood, cuvettes with a light path of 0.07 n were made. The cuvettes are similar to at described by Drabkin & Austin (1935), except at they were made to fit into an ordinary vette holder of the Beckman D. U. spectrophotoeter.[1]

For details of construction see legend of Fig. 2.

TECHNIQUE

The syringes with the heparinized blood samples are sealed with mercury, and the nozzles are closed with rubber tubes, stoppered with metal rods. For hemolysis a 3 per cent saponin solution (Merck)

[1] The cuvettes were made according to our design by the German firm: Dr. F. A. Wöhler Optische Fabrik, Wollfsangerstraße 12, Kassel.

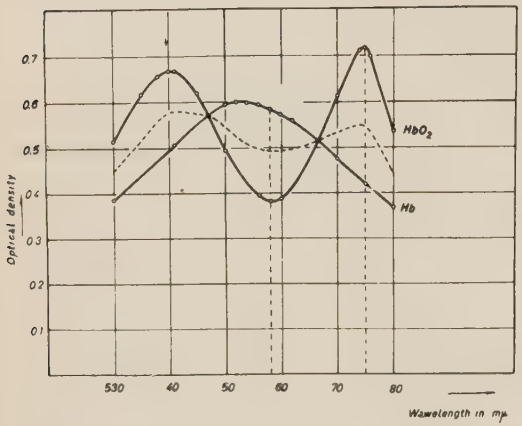

Fig. 1. Absorbtion curves for hemoglobin and oxyhemoglobin. Dotted curve, mixture of hemoglobin and oxyhemoglobin. The optical densities are read in hemolyzed blood. Light-path 0.07 mm.

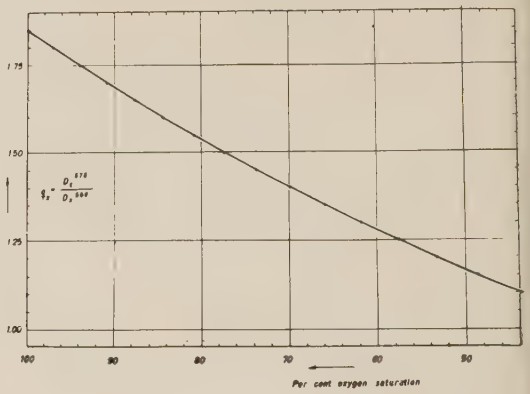

Fig. 3. Relationship between $q_x = D_x^{575} / D_x^{558}$, and per cent oxygen saturation $= (1 - X)\ 100$. Calculated from equation 2. $q_1 = 1.85$, $q_2 = 0.72$, and $r = 0.59$.

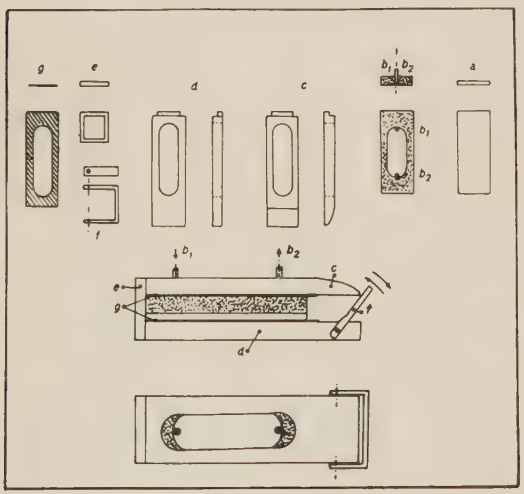

Fig. 2. The different parts of the cell.

The cover plate a, and the chamber plate b are made of silica. The cuvette chamber is ground into b to a depth of 0.07 mm. At each end of the chamber there are 0.5 mm wide borings vertically through the chamber plate, into which 6 mm long, 0.5 mm thick steel tubes (Record canulas No. 16) are fitted by means of a resinous fixative (b_1 and b_2).

is injected into the syringe through the rubber tube. For 5 ml blood 0.3 ml saponin solution suffices. The syringe is then furnished with a canula which is connected to the entry tube of the cuvette by means of a polyethylene tube. The chamber is easily filled by applying gentle pressure on the piston. We usually count 20 drops falling from the exit tube, as a thorough flushing of the chamber is advisable. The optical densities at 558 and 575 millimicrons are then determined in the Beckman spectrophotometer. The value of q is calculated, and the per cent oxygen saturation is read directly from the curve presented in Fig. 3.

The cuvette holder, c and d, is made of stainless steel, and held together by means of e and f. e is soldered to d, while f may be pivoted on an axis. The steel surfaces contacting the glass parts are covered by thin rubber sheets. The cell is assembled in the following order: Part d, part a, part b, the thin end of c is put into e, and the different parts are pressed together by swinging f over the curved end of c into vertical position.

RESULTS

The constants in equation 2, q_1, q_2 and r, re determined in several samples of blood, secure reliable values for the calculation oxygen saturation according to equation nd for the construction of the curve pre-nted in Fig. 3. Hemolyzed blood was uilibrated with air in a tonometer for 10 nutes, the cuvette was filled with part of blood, and the optical densities at 558 d 575 millimicrons were determined. To rest of the blood a few crystals of sodium hionite were added, the blood was care-ly stirred with a glass rod, and filled into same cuvette for determination of the tical densities at the same wavelengths. e cuvette was not disassembeled between two measurements, as this may lead to all changes in the light path. The deoxy-nated blood did however easily replace the ygenated blood in the cuvette.

The obtained values for q_1, q_2 and r are esented in Table I.

Table I. *The constants in equation 2, termined by measuring the optical den-ies of wholly oxygenated and deoxygenated hemolyzed blood at 558 and 575 millimicrons.*

	Number of determi-nations	Average	S. D.
$= D^{575}_{HbO_2} / D^{558}_{HbO_2}$	23	1.854	0.014
$= D^{575}_{Hb} / D^{558}_{Hb}$	10	0.718	0.005
$= D^{575}_{Hb} / D^{575}_{HbO_2}$	10	0.591	0.006

During a period of 3 months more than 90 parallel analyses were carried out according to the manometric and the spectrophotometric technique. The parellel samples were collected in 2 different syringes and analyzed as soon as possible. In 3 groups of samples, originating from 3 cardiac catetherizations, one or more determinations by the manometric method were obviously wrong, as they were either absurdly low, or in conflict with clinical evidence. In these cases the spectrophotometric technique yielded sensible results. These groups are not included in the presented material, which then consists of 82 parellel determinations.

The results are presented in Fig. 4.

The correlation coefficient was calculated and found to be 0.977. The point of intersection of the regression lines was: 80.23 per cent oxygen saturation (manometric method) and 83.47 per cent oxyhemoglobin (spectrophotometric method). Thus, the spectrophotometric method gave approximately 4 per cent higher results than the manometric method. At high levels of oxygen saturation the difference becomes less, as the physically dissolved oxygen becomes more important at high oxygen tensions, and this is not determined by the spectrophotometric method.

In a tonometer an amount of 250 ml blood was collected, and oxygen was added until a suitable saturation was obtained. Twenty syringes were then rapidly filled with 5 ml portions of blood, while the tonometer was occasionally rotated to prevent sedimentation of the erythrocytes. The syringes were sealed with mercury as described, and placed in ice-water. Ten samples were then analyzed by each of the methods. The results are presented in Table II.

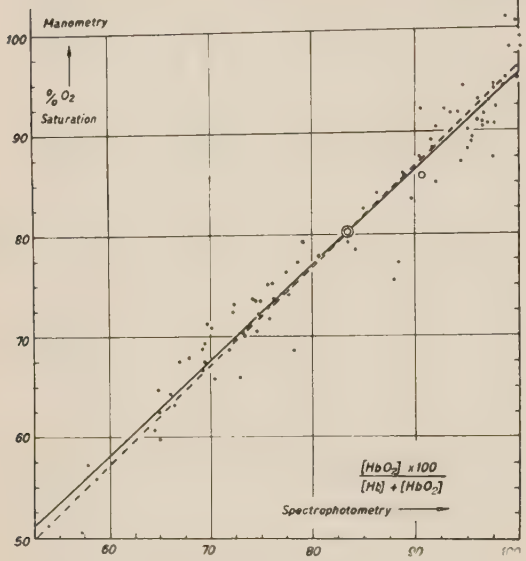

Fig. 4. Comparison of spectrophotometric and manometric determinations of oxygen saturation in parallel samples.
——y/x regression line. - - - x/y regression line.
⊙ point of intersection of the regression lines.
○ average values of 10 determinations by each method in blood from the same pool (Tab. II).

The average arterial oxygen saturation in normal subjects was reported by Storstein (1952) in this laboratory to be 94.7 per cent oxygen saturation. In 10 normal subjects we found an average arterial oxygen saturation of 98.0 ± 1.58 per cent by the spectrophotometric method.

DISCUSSION

The method presented above is based on the ingenious method of Drabkin and Schmidt (1945), but is simplified by measuring only the relative density in the unknown sample at two different wavelengths, according to a well known principle (Lambrechts, Lefevre & Cornil, 1942; Zijlstra, 1953).

Table II. *Comparison of the manometric and the spectrophotometric method for the determination of hemoglobin oxygen saturation in blood. Samples taken from the same pool, and analyzed as soon as possible.*

Per cent oxygen saturation.		
	Manometric method	Spectrophotometric mehod
	86.3	91.2
	85.3	90.8
	85.7	90.9
	86.2	90.9
	86.1	91.7
	85.8	91.0
	85.9	90.8
	85.8	91.3
	85.1	89.9
	84.8	90.7
Average	85.70	90.92
S.D.	0.49	0.47

Working with only slightly diluted blood, the accuracy of the measurements will suffer if the light path is too long. To our knowledge, only Drabkin and Austin (1935) have realy solved this problem, by making a cuvette with ground-in chamber and a light path of 0.07 mm. With this cuvette optical densities of suitable magnitude are obtained at 575 millimicrons, and measurements of great accuracy may be made.

The cuvette described by us has a light path of 0.07 mm (approximately). It is easily filled and air contamination is eliminated by the technique employed. The cuvette is not very expensive (approximately 100 DM) and may consequently be ordered in triplicate, making possible the full utilization of the Beckman spectrophotometer. Moreever it may easily be modified for the use of other types of spectrophotometers.

The speed of operation in determination oxygen saturation is comparable to that ordinary spectrophotometric analysis, and very favorably compared to that of the nometric method. A series of 10 samples uires in our hands 8 to 9 hours for the ermination of the oxygen saturation ording to the manometric method. The ne number of samples are by the spectro-otometric method easily analyzed in one l a half hours. Also, operators easily rn the new method, while rather tedious ining is necessary for the manometric nique.

The volume of blood needed is small. We e made no attempt to reduce the volume the blood samples below 2 ml, but it is ious that less than that is needed, as the ume of the cell and tubes is less than 1 ml.

The determinations according to the spec-photometric method are very accurate. the results presented in Table II, the ndard deviations in the spectrophotometric l the manometric determinations were ctically identical. This confirms our neral impression that the two methods about equally accurate.

The constants q_1, q_2 and r in equation may be determined with great accuracy e Table I). Thus, errors resulting from ccurate determinations of the constants easily avoided. When the oxygen satura-n is high and great accuracy is required, should, however, be determined in wholly ygenated blood from the sample.

Physically dissolved oxygen is not deter-ned by the spectrophotometric method. oxygen breathing the saturation may be more than 100 per cent. In such cases the manometric method should of course be used.

The average value for arterial oxygen saturation in normal individuals, found by the spectrophotometric method, is 98.0 per cent and 3.3 per cent higher than the value reported by Storstein (1952). 98.0 per cent oxygen saturation is in good agreement with the value now considered to be the correct one (Drabkin & Schmidt, 1945).

SUMMARY

1) A rapid and accurate method for spectrophotometric determination of oxygen saturation in slightly diluted, hemolyzed blood is described.

2) A cuvette with a light path of 0.07 mm, similar to that described by Drabkin and Austin (1935) was made. The cuvette is inexpencive, and convenient for routine use.

3) Eighty-two parallel samples have been analyzed by the spectrophotometric and the manometric method (Van Slyke/Neill). The correlation between the results was good, but the values obtained by the spectrophotometric method vere approximately 4 per cent higher than those found by the Van Slyke apparatus.

4) The two methods were found to be about equally accurate.

5) Less than 25 per cent of the time required by the manometric method is needed for the same number of determinations by the spectrophotometric method.

6) The average hemoglobin oxygen saturation in arterial blood from normal subjects was found to be 98.0 per cent by the spectrophotometric method.

ACKNOWLEDGMENT

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The members of the staff at the Medical Department Haukeland Sykehus are thanked for their cooperation.

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OX FOR REFRIGERATION AND REWARMING IN ANIMAL EXPERIMENTATION AND HUMAN SURGERY

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A good view of the surgical field is a dition for every branch of advanced gery. Hitherto the surgeon working hin the interior of the heart has been ied this favor, for instance when per- ming commissurotomy for mitral stenosis closing an interatrial septal defect. Not il recent years has experimental surgical earch indicated methods permitting the rt to be temporarily excluded from the culation. These methods are principally two different kinds:

) The function of the heart is substi- ed by a heart-lung machine or 2) the tabolism of the body, especially the oxy- consumption, is depressed by means of ering the body temperature to such an ent that the circulation of blood may be porarily stopped without any irreversible nage to the cells. After experimental ls, particularly by Bigelow, Lindsay & eenwood; Bigelow, Callaghan & Hopps; gelow & McBirnie (1950—53), the tech- ue of hypothermia has recently been plied in clinical work (Lewis & Taufic 53, Swan, Zeavin, Blount & Virtue 1953; ley, Cookson, Downing & Neptune 1954). n these experiments and operations the hnique for inducing the hypothermic state l rewarming attracted considerable inter- est. Several different methods for lowering body temperature have been employed. During operations on the heart, the body temperature for different reasons should be kept between +24° and +28° C, for more time-consuming interventions probably somewhat lower. All the methods for inducing hypothermia have their pros and cons, and shall here be briefly discussed.

Packing of the patient in ice or *bathing in ice-cold water* was originally used by Smith & Fay (1940) as a therapeutic adjunct to patients with advanced cancer suffering from intractable pain. Later this method has been used for refrigeration before and during operation. The method is simple, cheap and permits a rather rapid cooling of the body. However, it is very inconvenient to handle large amounts of wet and splashing ice or ice-water in the operating theatre. The possibility of maceration of the skin is obvious. Using this method rewarming of the body cannot be carried out directly; as a rule it has been executed by immersing the patient in a hot bath, a practice that is as unwieldly as it is insanitary.

Wrapping of the body in wet blankets has about the same advantages and drawbacks as packing in ice. A further step forward

was taken by using *blankets and/or matresses with cooling coils* (Bigelow, Lindsay & Greenwood 1950; Inglis, Biffen & d'Abreu, 1954). The splashing of the ice or ice-water is eliminated by this method. On the other hand there will be an insulating layer of air between patient and blanket, rendering it more difficult to refrigerate or rewarm the body. Another disadvantage is the difficulty of covering the body properly with these rather stiff blankets. In preliminary experiments in our laboratory we tried a "figure-made" suit on dogs, thus diminishing the latter inconvenience.[1] The insulating layer of air still persists, however.

By using the body's own vessels as a cooling coil it is possible to obtain a rather rapid refrigeration. A part of the circulation is exteriorized by means of an arterio-venous communication outside the body including a coil immersed in a cold medium (Delorme, 1953 and others). As a rule this arrangement requires a pump for the blood. Juvenelle combines this method of refrigeration with immersion of the whole body in a cold bath and also uses a heart-lung machine for oxygenation of the exteriorized blood. By this technique it is possible to reach very low temperatures in relatively short time. According to our opinion, which however is not founded on own experiments, this arrangement adds the inconvenience of an arterio-venous shunt and pump-traumatization of the blood to that of hypothermia itself. Furthermore, heparin or another anticoagulant must be given which is disadvantageous in the surgical work.

[1] We are indebted to Trelleborgs Gummifabriks AB, Trelleborg, Sweden, for kind cooperation in manufacturing this "costume".

In France, Huguenard & Laborit have worked out a pharmacological method for inducing hypothermia, *"hibernation artificielle"*. The body temperature is lowered solely by pharmaceutical means; sometimes they use a small amount of external refrigeration of the precordium and the abdomen. This method has been described in detail in several papers, most recently in a monography "Pratique de l'hibernothérapie" (Masson & Cie, Paris, 1954). The main advantage of the method is its simplicity — it requires no special equipment and can be accomplished by every experienced anesthetist. The French workers practice, however, a rather puzzling polypharmacy and the desirable control of this potentiated anesthesia must be questioned. A definite disadvantage of the method is the long rewarming period (often several days), which calls for permanent close and experienced supervision, preferably in a special post-operative ward.

After perusal of the pertinent literature and own preliminary experiments with hypothermia on dogs we discussed the possibility of lowering the body temperature in a more simple, reliable and comfortable manner. A hint of a possible way was found in the description of a refrigerator built by Adams-Ray & Persson (1953), originally designed for the study of certain cold injuries.

The following demands should be made upon an apparatus, designed for refrigeration of an organism which is to be subjected to surgery:

1) Sufficient capacity for lowering the body temperature to +20°—+25° C in reasonable time.

2) Possibility to rewarm the body without moving it and without delay.

3) Avoidance of extra-corporal circu-
ion.
4) Cleanliness.
5) Avoidance of cold injuries to the skin.
6) Possibility for immediate and con-
uous control of the patient with electro-
diography, electroencephalography, elec-
al recording of rectal, oesophageal and
n temperatures etc. throughout the per.od
hypothermia.
7) Possibility for simple and comfortable
inistration of orthodox inhalation and
. anesthetics and comfortable access to a
n for the administration of i. v. drips.
On the other hand the necessity of good
ess to the surgical field during the period
cooling may be ignored since a cold
ganism as a rule remains in the hypo-
rmic state for 2—4 hours provided it is
subjected to active rewarming. During
s period the patient can be removed from
refrigerator and put on an ordinary
erating table. For this however, is required
8) Possibility for rapid and easy trans-
rt of the patient to the operating table.
Proceeding from these demands we have
signed an apparatus, principally a modi-
ation of that of Adams-Ray & Persson.[1]
e refrigerator has passed experimental
ts on dogs to our complete satisfication.

Description of the refrigerator

The refrigerator is made in two parts, one
t containing the refrigeration equipment,
ating coils and a fan, the other part fitted with a stretcher intended for the patient or an experimental animal (Fig. 1) and the air-return-channel. For the first manufactured box we chose such dimensions as to be suitable for children 12 years of age or less and for dogs. The external measurements are $2005 \times 950 \times 605$ mm. The stretcher compartment is mounted on a base of steel pipes. The machinery as well as the stretcher part is mounted on rubber casters making it possible to transport both parts coupled together as well as separately. The two parts are easily coupled together by means of 4 clasps. The possibility of dividing the whole equipment into two parts serves a dual purpose: the stretcher compartment can be replaced by a bigger one designed for adults, and moreover the cleaning of the apparatus is facilitated.

The outer lining of the refrigerator consists of steel sheets with a finish of white synthetic enamel except for the top which is covered with stainless steel. Even the inner lining is stainless steel. The insulating layer consists of 60 mm "Wellit". On the top of the stretcher compartment is a 550×550 mm readily removable glass window. A door is fixed to the end of this compartment for handling the stretcher. The stretcher consists of a frame-work of stainless steel pipes with a network of 4 mm plastic cords. The stretcher is furthermore fitted with small wheels adjusted for rails mounted inside the box. It can be looked in a position halfway out of the box if necessary (Fig. 2).

For connections to recording instruments, such as electrocardiograph or electroencephalograph, the box is equipped with 15 electrical connectors on the side. At the same panel will be found 5 channels for drip tubes

[1] We are indebted to Damgaards Maskin AB, lmö, Sweden, who have been of great assistanse us during our experiments, and who have finally structed the refrigerator in accordance with our iderata and to our entire satisfaction.

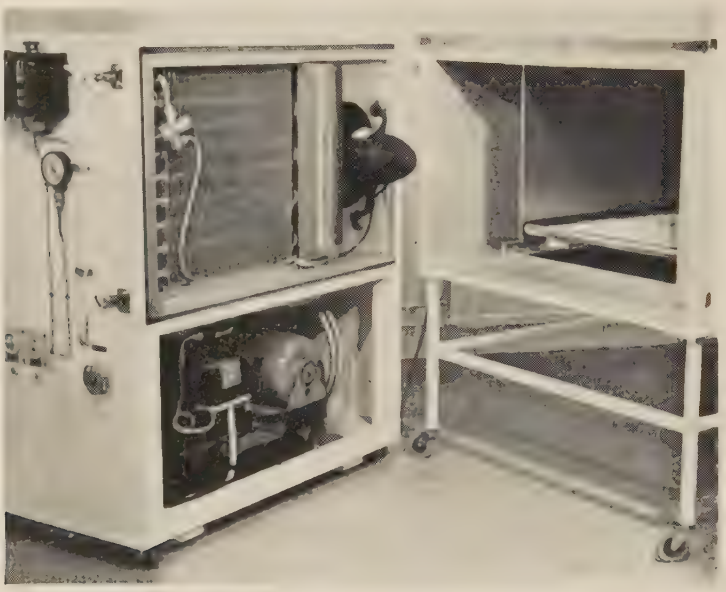

Fig. 1. The two box compartments uncoupled showing the compressor, fan and stretcher.

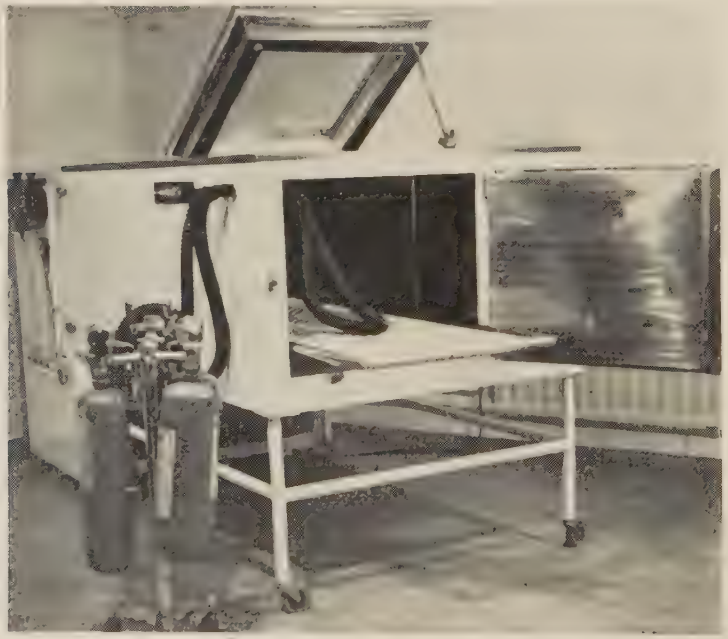

Fig. 2. Total view, showing the door for handling the stretcher and the top window semi-opened.

d 2 pipes for anesthesia tubing of standard mensions.

The refrigeration is produced by a Frig- ire air-cooled condensing unit with a pacity of about 100 kcal/h at a refrigerant nperature of ± 0° C. Heat losses such as at leaks, fanmotor heat-eqvivalent, human dy metabolism heat, heat gains by treat- nt and other losses are calculated to about 0 kcal/h, leaving approximately 600 kcal/h r lowering the body temperature at ± 0° C frigerant temperature. At +10° C refr. nperature, corresponding to — 2° C air nperature, the remaining refrigeration nperature for lowering the body tempera- re will be approximately 300 kcal/h. By ans of a fan the cooled air is brought to culate with an approximate velocity of 5 6 m/sec. For rewarming the body the box equipped with two heating coils, each with capacity of 450 Watt totalling 770 kcal/h. Refrigeration as well as heating is con- olled by 2 thermostates, placed on the out- le of the machinery compartment and adily adjustable during the process. The ctrical supply for compressor and heaters for 220 v 50 cyc. A. C.

Experiments on dogs

The refrigerator has been tested in a ries of experiments on dogs. In order to ake conditions as similar as possible as ose to be expected when refrigerating man beings, we cut the hairs of the dogs nd made an extensive epilation using ba- um sulphide. Dogs weighing 16 to 26 kg ere used. After an initial dose of 5 per nt pentothal sodium i.v. to permit handling e dog, tracheal intubation anesthesia was aintained with ether — N_2O—O_2 in semi-open circuit. At body temperature below 30° C only 100 per cent oxygen was ad- minstered and below +28° C controlled respiration was maintained regardless of the animals' respiratory and cardiac condition.

Using an air temperature of +1° C to — 6° C in the refrigerator, we lowered body temperatures to between +21° C and 25° C. During the progress of the series we grew bolder lowering the temperature in the box for every new experiment.

As a rule the duration of the cooling period varied between 1.7 and 3 hours. The difference may be explained by

1) individual sensibility to cold
2) degree of epilation
3) varying temperature in the box.

Generally, we now reckon the cooling time for a 20 kg dog (shaved and epilated) to 1.5—2 hours at a box temperature of ±0° C to — 2° C. Probably human beings of corresponding weight will reach low body temperature comparatively faster, due to the almost complete lack of insulating hair.

All dogs have been carefully observed during and after experiments with special regard to the possibility of cold injuries to the skin. In no case have we been able to record any damage.

Body temperature was controlled by means of thermocouple rectal and skin leads. Electrocardiographic records were obtained and blood-samples collected from a polyethylene catheter in the inferior caval vein throughout the experiment. The results of these investigations will be published later in a separate paper.

The course of a typical experiment is shown in Fig. 3.

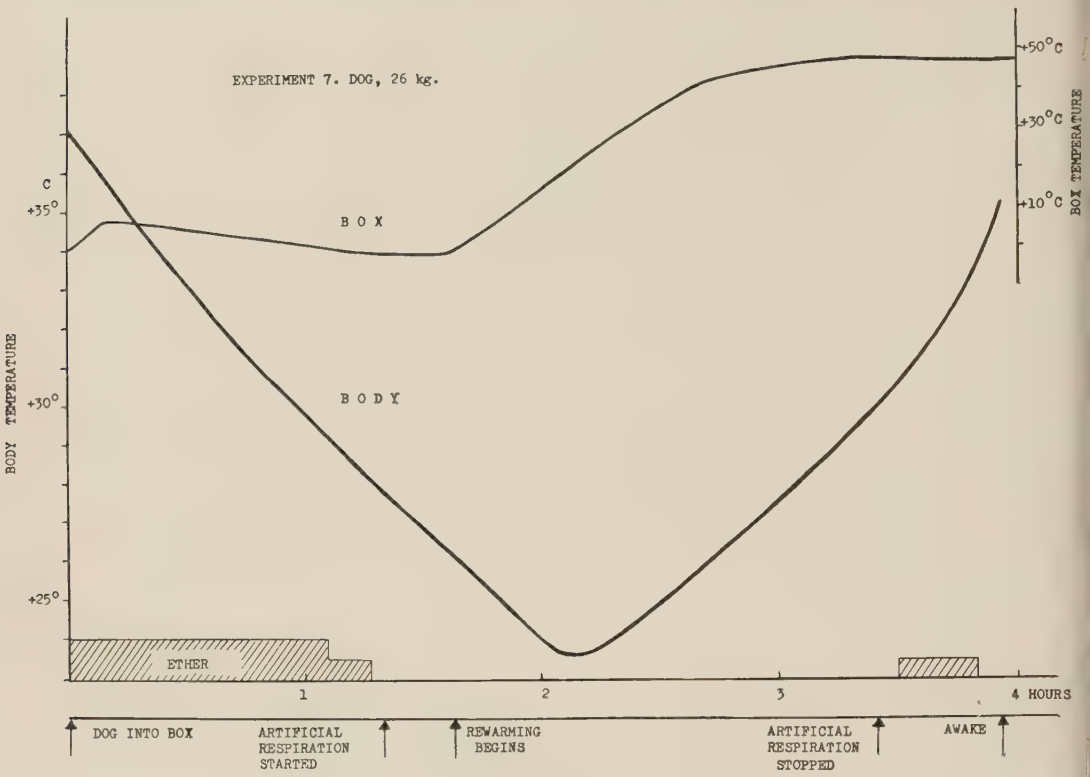

Fig. 3. The course of a typical experiment. Dog 26 kg.

SUMMARY

Common methods for cooling patients for cardiac surgery are briefly discussed. A refrigerator unit for human beings and animals, also fitted with heating radiators for rewarming, is presented. Some experiments with dogs, cooled to about +20° C, are reported.

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THE TITRATION OF HEPARIN WITH PROTAMINE

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Fischer (1935), Fischer & Astrup (1935) and Jaques (1943) have demonstrated that heparin is a strong acid forming salts with proteins. Chargaff & Olson (1938) showed that the compound of heparin and protamine is so nearly undissociated that the anticoagulant action of heparin is blocked. Under heparin treatment and in other cases where the heparin content of the blood is increased, this can be determined by titrating with protamine.

According to the method described by Jaques & Waters (1941), varying quantities of protamine are added to whole blood samples. The use of non-preserved blood involves that titration must be done very soon after the sample is taken. Moreover it is difficult to observe the coagulation simultaneously in several tubes, and the method is sensitive to greater or smaller admixture of thromboplastin, inherent in venipuncture. Allen, Moulder, Elghammer, Grossman, McKeen, Sanderson, Egner & Crosby (1949), describe a modification of the method, in which the titer is read at the end of an hour. Ullrich & Cronkite (1947) and LeRoy, Halpern & Dolkart (1950) in their method employ oxalated or citrated plasma samples, add varying amounts of protamine and equal amounts of thromboplastin and start coagulation by adding $CaCl_2$, whereafter the clotting time is determined. This method is also sensitive to variations in the amount of thromboplastin, whether due to the admixture of tissue thromboplastin or to the limited stability properties of thromboplastin preparations.

Here is presented a description of a titration of heparin with protamine in which the addition of thrombin is employed both for releasing the coagulation and as an indicator of the titration. The addition of thrombin assures that the titration is not affected by a larger or smaller amount of thromboplastin in the plasma samples. The antithrombin effect of heparin is utilized for determining the equivalence point, as a small excess of heparin in proportion to protamine has a marked effect on the coagulation time.

TECHNIQUE

Reagents:
0.9 per cent NaCl-solution.
40 per cent sodium citrate solution
and the following stock solutions prepared in 0.9 per cent NaCl solution:
heparin 400 γ/ml.
protamine sulphate 1 mg/ml.
thrombin (Hoffmann la Roche) 1 mg/ml.

By venipuncture withdraw 10 ml blood and transfer it directly from the syringe into a centri-

ge tube containing 0.1 ml 40 per cent sodium rate solution. Mix the sample and centrifuge, n remove the plasma with a Pasteur pipette. ansfer 0.5 ml plasma to a test tube with an ide diameter of 7 mm and a length of 70 mm. ld 0.3—0.7 ml of a diluted protamine sulphate lution and then 0.2 ml diluted thrombin solution. mediately after addition of the thrombin start stopwatch, close the tube with a paraffined cork d tilt it continuously until coagulation occurs. ccess of protamine will produce a short clotting ne as shown in Fig. 1, independent of the excess nount. If there is a protamine deficit — *i. e.* cess of heparin — the effect will be a prolonged otting time, due to the antithrombin effect of parin, even very small differences in the quantity free heparin involving great variations in the otting time. Utilizing 5 to 7 plasma samples, ml each, and varying amounts of protamine, equivalence is determined, corresponding to the allest amount of protamine that gives a short otting time (see Fig. 1).

The equivalence ratio between protamine d heparin:

$$= \frac{\gamma \text{ protamine sulphate}}{\gamma \text{ heparin}}$$ is about 1.3 but ries with the protamine sulphate employed. order to avoid marked variations in the olume of the reaction mixture, the con- ntration of the protamine sulphate solution varied according to the expected heparin ntent (see ex.). With a heparin content of out 10 γ/ml plasma, a protamine sulphate lution of about 15 γ/ml will be suitable.

As a guide in the choice of a suitable lution of thrombin for the assay it may stated that 0.2 ml solution should give a otting time lying between 10 and 20 seconds ith 0.5 ml normal plasma.

The equivalence ratio is determined from e difference observed when titrating rmal plasma samples after addition of nown quantities of heparin, *e. g.* 10 and γ hep./ml plasma.

If it is found that a plasma sample of which the heparin content is to be determined contains less than 5 γ/ml, it is of advantage to add a known amount of heparin in vitro.

In a series of dog experiments the titres were determined on two samples taken one immediately after the other by venipuncture. In these titrations the standard deviation of a single determination was 15 per cent.

It is essential that all glass surfaces are thoroughly clean and that the test tubes employed have equal diameters. It has been found that the addition of NaCl solution to compensate for variations in volume of protamine solution is unnecessary. The analyses can be made at room temperature, though stock solutions should be kept cold.

The earlier methods of titration give increasing clotting times when excess of protamine is increased and therefore a minimum in clotting time at equivalence is obtained. In the method here described excess of protamine does not alter the clotting time. According to Chargaff (1938) the explanation is that the anticoagulant effect of protamine is not an antithrombin effect. This constancy of the clotting time with excess of protamine makes the equivalence point less well defined, theoretically, but in practice it is an advantage, because a prolonged coagulation time unequivocally shows that more protamine must be added, and therefore the equivalence point is found more quickly and with the use of a smaller quantity of plasma.

The method has been applied in control of sublingual heparin dosage (Refn & Raaschou 1952) and subcutaneous application of heparin (Raaschou, Refn & Vestergaard 1954).

EXAMPLE

Choice of thrombin amount :

γ thrombin in 0.2 ml	ml plasma	cl.time sec.
20	0.5	18.0—18 5
30	0.5	16.0—14 0
40	0.5	10.0— 9.5

The choice fell upon 20 γ thrombin in 0.2 ml.

Determination of equivalence ratio :
Normal plasma with 10γ heparin/ml added

ml heparin-plasma	ml protamine-sulph. 16 γ/ml	ml thrombin 100 γ/ml	cl.time sec.
0.5	0.500	0.2	32
0.5	0.425	0.2	33
0.5	0.400	0.2	39
0.5	0.375	0.2	69

0.425 ml $\times$ 16 γ/ml=6.8 γ protamine sulphate

Normal plasma with 20 γ heparin/ml added

ml heparin-plasma	ml protamine-sulph. 32 γ/ml.	ml thrombin 100 γ/ml	cl.time sec.
0.5	0.500	0.2	31
0.5	0.425	0.2	31
0.5	0.400	0.2	38
0.5	0.375	0.2	47

0.425 ml $\times$ 32 γ/ml=13.6 γ protamine

$$\varphi = \frac{13.6—6.8}{10 — 5} = 1.36$$

TITRATION

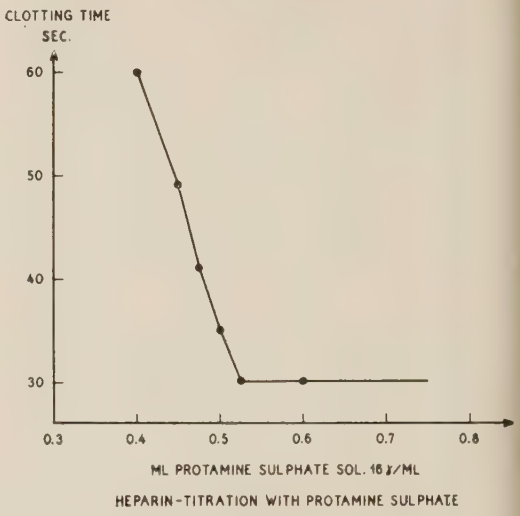

Fig. 1.

4 ml human plasma 4 hrs. after subcutaneous injection of 30.000 i. u. heparin

ml plasma	ml protamine-sulph. 16γ/ml	ml thrombin 100γ/ml	cl.time sec.
0.5	0.600	0.2	30
0.5	0.525	0.2	30
0.5	0.500	0.2	35
0.5	0.475	0.2	41
0.5	0.450	0.2	49
0.5	0.400	0.2	60

Fig. 1 shows equivalence at 0.525 $\times$ 16 $\times$ 2=16.8 γ protamine sulphate.

I.e. $\frac{16.8}{1.36}$ =12 γ hep/ml.

SUMMARY

A method is described for titrating heparin with protamine, in which the addition of thrombin is employed both for releasing coagulation and for determining the equivalence point.

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DETERMINATION OF ANOMALOUS VISCOSITY IN PATHOLOGICAL JOINT FLUIDS

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The hyaluronic acid of pathological joint fluids has in recent years been the subject of considerable attention (see reviews by Ropes, Robertson, Rossmeisl, Peabody & Bauer, 1947; Ropes & Bauer, 1953; and Sundblad, 1953). In studying the degradation of the polysaccharide encountered in such fluids, viscosity/concentration relations have usually been employed. Such methods are, however, complicated by the pronounced non-Newtonian behaviour of the viscosity.

This difficulty is overcome in the method of Sundblad (1953), where the mean polymerization of the hyaluronic acid is characterized by the intrinsic viscosity, measured in capillary viscometers of fixed dimensions.

On the other hand, the flow anomalies provide an independent method of estimating the degree of degradation of the synovial polysaccharide. Ogston & Stanier (1952) have shown that the variation of the viscosity with velocity gradient, as measured in a modidified Couette viscometer (Ogston & Stanier, 1950), is a more sensitive test of degradation than the viscosity at a fixed gradient. These authors have also suggested a procedure (Ogston & Stanier, 1953) by which the strong dependence of the anomalous viscosity on concentration may be eliminated. The degree of non-Newtonian variation of the viscosity is thus represented by plotting $\frac{\eta_0}{\eta_{50}}$ against η_0 (η_0 = relative viscosity extrapolated to zero rate of shear, η_{50} = relative viscosity at 50 sec^{-1}). By comparing the position of the point $\frac{\eta_0}{\eta_{50}}$, η_0 for a pathological fluid with the curve obtained for normal fluids, information about the degree of degradation in the sample may be obtained without the need for any direct measurement of concentration.

The main disadvantage of the method of Ogston & Stanier is that the Couette viscometer is unsuited to routine determinations in pathological fluids, especially when only small amounts of test sample are available.

Sundblad (1953) has suggested the use of capillary viscometers for similar determinations of anomalous viscosity. In such viscometers, however, neither the value of η_∞ (*i. e.* the viscosity at infinite rate of shear) nor that of η_0 (*i. e.* the viscosity at zero rate of shear) can easily be measured in synovial fluid. From flow diagrams plotted as log $[\eta]$ ($[\eta]$ = intrinsic viscosity) against shearing stress, a convenient measure of the degree of flow anomaly can, however, be obtained as the difference between the values of log $[\eta]$ at two chosen

d stresses. Both for hyaluronate solu-
s and joint fluids the degree of ano-
ous viscosity measured in this way was
nd to be roughly proportional to the in-
sic viscosity at a fixed (high) stress,
cating that both methods might be used
btain a quantitative measure of the de-
e of polymerization of the hyaluronic
l.

he aim of the present study has been
nvestigate whether similar determinations
nomalous viscosity can be used to study
nges in the synovial polysaccharide oc-
ring in connexion with hydrocortisone
tment of rheumatoid arthritis (see the
mpanying paper, page 295.

ecently another method of characterizing
viscous behavior of pathological syn-
l fluid has been proposed by Scott Blair,
liams, Fletcher & Markham (1954). In
the relative viscosity at a fixed stress
easured in a specially designed capillary
ometer; in addition the rate at which
viscosity changes with changing stress is
rmined.

he shear-dependence of the relative
osity will vary, however, both with con-
tration and degree of polymerization of
hyaluronic acid; the authors suggest that
ratio between the stress anomaly and the
osity at a chosen stress might be of
ificance in judging changes in patholo-
l fluids.

EXPERIMENTAL

Most of the fluids tested were obtained
m the knee joints of patients with rheu-
toid arthritis, some of which were under
tment with hydrocortisone (see the ac-
npanying paper). The samples were handled under sterile conditions, and were stored at — 20° C if immediate analysis was not possible. Fibrin clots were removed by centrifugation and the supernatant diluted with phosphate-NaCl of pH 7.0 and ionic strength 0.20 (0.08 as phosphate and 0.12 as NaCl) before performing the tests.

The hyaluronic acid content of the fluids was calculated from the glucosamine component determined according to the method of Sundblad (1953).

Viscosity was measured at 37° C in an Ostwald viscometer of the semimicro type designed by Cannon & Fenske (1938). The viscometer required about 0.5 ml of test sample, and had an efflux time of 80 sec. for water at 37° C. The stress at the wall of the capilllary, τ, calculated as $\tau = \frac{P \cdot r}{2l}$, was 7.0 dynes per cm² (P is the pressure difference across the capillary, r its radius, and l its length). One side tube of the viscometer could be connected with a manostat, which could maintain constant pressures within the range 2—30 cm of water. Determinations were usually made at an extra pressure of 20 cm water, giving a value of τ of 17.0 dynes per cm².

η_{rel} (relative viscosity) was calculated relative to the viscosity of the salt-buffer mixture used to dilute the samples, so that η_{rel} was within the range of about 2.5—10 at 37° C. η_{sp} $(= \eta_{rel} - 1)$ is the specific viscosity. A correction for protein viscosity has to be made in calculating the specific viscosity of the hyaluronic acid in joint fluids. This correction was determined by incubating the fluids with 100 VU hyaluronidase per ml of test sample for about

20 hours at 37° C, a drop of toluene added (for definition of the unit VU, see Sundblad, 1953). The intrinsic viscosity, $[\eta]$, *i. e.* the limiting value of the ratio $\frac{\eta_{sp}}{c}$ (c = concentration in g per 100 ml) at zero concentration, was calculated according to the formula (Sundblad, 1953): $[\eta] = \frac{\frac{\eta_{sp}}{c}}{1+0.16\,\eta_{sp}}$ (1), valid for values of η_{sp} up to about 15 at 37° C. This formula was shown to hold for determinations at different stresses (tested both in different-sized capillaries and at varying pressures in the same capillary) provided that the different viscosity determinations of each dilution curve had been made at the same stress. The values of $\frac{1+0.16\,\eta_{sp}}{\eta_{sp}}$ corresponding to the values of η_{sp} were conveniently taken from graphs. The values of $[\eta]$ given refer to determinations at the stress 17.0 dynes per cm² unless otherwise stated.

RESULTS

Fig. 1 illustrates the flow anomalies in three samples of joint fluid containing hyaluronic acid of high, medium, and low degrees of polymerization, as measured by the intrinsic viscosity.

The anomalous viscosity, recorded as the ratio between the specific viscosities measured at two different pressures in the same viscometer (stresses of 7.0 and 17.0 dynes per cm²), is seen to vary both with concentration and degree of polymerization of the hyaluronic acid.

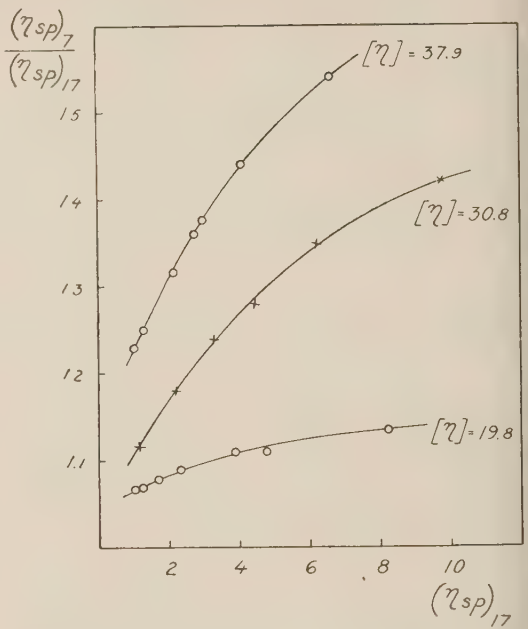

Fig. 1. Variation in the anomalous viscosity of synovial fluids with concentration and degree of polymerization of the hyaluronic acid. Specific viscosities were measured at 7 and 17 dynes per cm² in synovial fluids diluted to varying degrees with phosphate-NaCl of pH 7.0. Anomalous viscosity is expressed as $\frac{[\eta_{sp}]_7}{[\eta_{sp}]_{17}}$.

The values for $\log \frac{[\eta]_7}{[\eta]_{17}}$ may obviously be directly calculated from the values of η_{sp} according to eq. (1). By this procedure a quantitative measure of the flow anomalies is obtained; this measure is largely independent of concentration, as is shown by the data in Table I.

The values for $\log \frac{[\eta]_7}{[\eta]_{17}}$ are seen to be approximately constant over a wide range of viscosities. Only at very low viscosities (η_{sp} below 1—1.5) did the values tend to

Table I. *Anomalous viscosity of synovial fluids calculated as* $\log \frac{[\eta]_7}{[\eta]_{17}}$ *from dilution curves at stresses of 7.0 and 17.0 dynes per cm².*

η_{sp} = specific viscosity at 17 dynes per cm². The fluids were diluted with phosphate-NaCl of pH 7.0.

No. D_3		No. A_{27}		No. A_{26}		No. A_{49}		No. A_{29}	
sp	$\log \frac{[\eta]_7}{[\eta]_{17}}$	η_{sp}	$\log \frac{[\eta]_7}{[\eta]_{17}}$	η_{sp}	$\log \frac{[\eta]_7}{[\eta]_{17}}$	η_{sp}	$\log \frac{[\eta]_7}{[\eta]_{17}}$	η_{sp}	$\log \frac{[\eta]_7}{[\eta]_{17}}$
23	0.025	12.60	0.033	8.24	0.056	9.84	0.053	6.62	0.082
74	0.024	3.20	0.035	4.75	0.059	6.17	0.061	4.10	0.087
88	0.026	1.74	0.037	3.66	0.060	4.53	0.059	2.99	0.091
33	0.026	0.93	0.036	3.41	0.057	3.27	0.061	2.74	0.090
65	0.029	0.51	0.027	3.33	0.061	2.23	0.052	2.18	0.085
30	0.024	0.41	0.033	2.71	0.059	1.34	0.049	1.31	0.081
04	0.024			1.68	0.058			1.00	0.076
				1.29	0.046				

crease. It should be noted, however, that e experimental error was considerable in s range, especially at low degrees of ano- lous viscosity.

In the range of η_{sp} above 1.5 the value of $\frac{[\eta]_7}{[\eta]_{17}}$ could be measured with reasonable curacy. The error of a single determination s thus found to be ± 5.6 per cent, as cal- lated from 25 duplicate determinations at fferent degrees of dilution (η_{sp} between 5 and 10).

The polysaccharide molecule of synovial uid is considered to have a flexible long- ain structure (Ogston & Stanier, 1951). or a polyelectrolyte with this structure the scous anomaly may be expected to de- ease with increasing ionic strength, owing a coiling up of the molecule (Künzle, 49; Alexander & Hitch, 1952). The data Table II are in accordance with this. The fect on flow anomalies of varying the nic strength of the solvent is seen to be marked for the sample of high anomalous viscosity. The viscosity at high stress is relatively less influenced by variations in electrolyte concentration.

Table II. *Effect of electrolyte concentration on the anomalous viscosity and intrinsic viscosity of synovial fluids.*

Two samples of joint fluid were dialysed against phosphate-buffer of pH 7.0 and ionic strength 0.10 for 3 days. Equal volumes of buffer containing varying amounts of NaCl where then added. $[\eta]$ = intrinsic viscosity at 17 dynes per cm².

Ionic strength	Fluid No. A_{87}		Fluid No. A_{89}	
	Anomalous viscosity, $\log \frac{[\eta]_7}{[\eta]_{17}}$	$[\eta]$	Anomalous viscosity, $\log \frac{[\eta]_7}{[\eta]_{17}}$	$[\eta]$
0.10	0.074	37.7	0.061	33.9
0.15	0.061	35.0	0.050	32.0
0.20	0.053	33.8	0.049	29.8
0.25	0.045	32.9	0.044	27.8
0.30	0.043	33.2	0.038	26.6

Table III. *Anomalous viscosity of synovial fluid determined at different stresses.*

As a measure of the flow anomalies the value of $\log \frac{[\eta]_{P_1}}{[\eta]_{P_2}}$ calculated from determinations at P_1 and P_2 dynes per cm² was used.

P_1—P_2	5—15	6—16	7—17	8—18	9—19	10-20
$\log \frac{[\eta]_{P_1}}{[\eta]_{P_2}}$	0.088	0.080	0.080	0.073	0.067	0.067

Determinations of anomalous viscosity must accordingly be made at essentially constant ionic strength, but since often only small amounts of test sample (less than 0.5 ml) were available, dialysis of the samples against buffer-NaCl before the determination was inconvenient. By the procedure employed, slight variations in ionic strength may arise from varying proportions of buffer and test sample. Errors due to this will, however, be included in the experimental error given above, since this was calculated from duplicate determinations at varying degrees of dilution.

The stresses 7 and 17 dynes per cm² were arbitrarily chosen for practical purposes. A different range of stresses may naturally be used to obtain a relative measure of the viscous anomaly. As can be seen from Table III, the variation in degree of flow anomalies is comparatively slight in similar determinations over the range of stresses conveniently covered by the semimicro viscometers used.

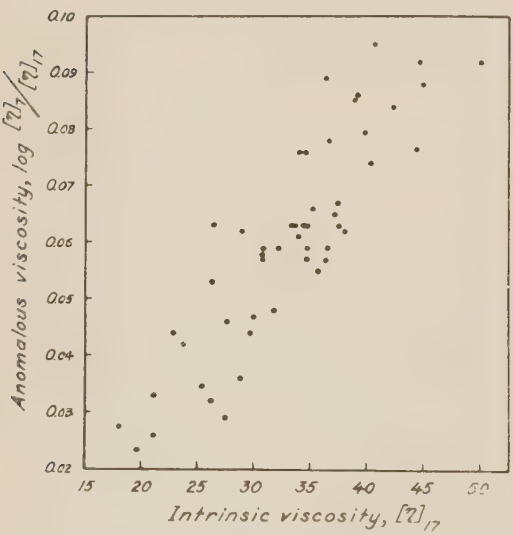

Fig. 2. Correlation between degre of anomalous viscosity and intrinsic viscosity in synovial fluids. $[\eta]_7$ and $[\eta]_{17}$ denote intrinsic viscosities measured at 7 and 17 dynes/cm². By the rank method, a correlation coefficient of $+ 0.87 \pm 0.14$ ($P < 0.001$) was found for the 50 fluids.

DISCUSSION

The results given in Fig. 2 indicate that determination both of anomalous viscosity and of intrinsic viscosity at a chosen stress may be used in estimating the degree of polymerization of hyaluronic acid in pathological fluids. The degree of correlation between the values is seen to be fairly high in the 50 fluids tested.

The correlation is probably weakened to some extent by variations in degree of polydispersity. As can be seen from Fig. 1, flow anomalies are marked, even at high dilutions, in fluids containing hyaluronic acid of high mean polymerization. The presence even of a small fraction of highly polymerized compounds in a fluid containing hyaluronic acid of low mean polymerization may therefore give rise to marked flow anomalies, whereas the intrinsic viscosity measured at high stress will be relatively less influenced.

t would seem that the degree of ano-ous viscosity may not be a reliable meas- of the mean polymerization in fluids taining highly degraded hyaluronic acid. rther, the data of Fig. 2 indicate the ential absence of flow anomalies in the ge of intrinsic viscosity below about 20 e also Sundblad, 1953).

On the other hand, the determination of malous viscosity seems to be a more sitive test than the determination of in-sic viscosity in detecting high-molecular tions in pathological fluids which may ear during treatment, e. g. as observed heumatoid arthritis following the use of rocortisone (Sundblad, Egelius & Jons- 1954).

oth methods may be used in studying luronic acid changes in pathological ds. By the procedure described a com-e analysis requires between 0.1 and ml, depending on the viscosity of the sample.

he advantage of the anomalous viscosity hod is that no determination of hyal-nic acid is needed. The method may efore prove to be of value as a clinical for degradation of hyaluronic acid in ological fluids. Such tests have been wn to be of diagnostic value in certain s of joint disturbance (see Ropes *et al.*, 7; Ropes & Bauer, 1953; Sundblad, 3).

inally, determinations of anomalous osity might well provide a useful tool for lies of hyaluronic acid changes when ted mucopolysaccharides are also present, in connective-tissue extracts. The pre-e of chondroitin sulphuric acid would interfere with the determination of hyaluronic acid concentration (and accordingly with the determination of $[\eta]$). On the other hand, chondroitin sulphuric acid has a much lower viscosity than hyaluronic acid (see Blix & Snellman, 1945), and would probably influence the degree of anomalous viscosity only insignificantly. (Of three preparations of chondroitin sulphuric acid tested, none showed measurable flow anomalies even at a concentration of 0.5 g per 100 ml).

SUMMARY

A method of determining the degree of anomalous viscosity in synovial fluid is described.

The method may be used for estimating the degree of degradation of hyaluronic acid in pathological fluids. No determination of hyaluronic acid is required.

ACKNOWLEDGMENT

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ACTION OF HYDROCORTISONE ON THE HYALURONIC ACID OF JOINT FLUIDS IN RHEUMATOID ARTHRITIS[1]

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The possible influence of ACTH or cortisone upon the mucopolysaccharide metabolism of connective tissue has been the subject of much study during recent years. As to the present the problem has commonly been approached indirectly by biological methods (spreading and permeability tests), few attempts at chemical investigation of the polysaccharides having been made.

Analysis of the hyaluronic acid in joint fluids might provide a clue to this question. It has been shown (Ragan & Meyer, 1949; Sundblad, 1950, 1953) that changes occur in the hyaluronic acid of joint fluids in rheumatoid arthritis, these changes having been described as due to overproduction of incompletely polymerized polysaccharide. Such changes are, however, by no means specific for rheumatoid arthritis, but occur in a variety of joint diseases, although usually to a lesser extent (see the review by Sundblad, 1953).

It is known that the viscosity of joint fluid increases following the systemic administration of ACTH, cortisone, and hydrocortisone, and on intra-articular administration of hydrocortisone. This increase might be due to either quantitative or qualitative changes in the polysaccharide, since both types of change influence the fluid viscosity. It has been stated (Clark, Ropes & Bauer, 1950; Bywaters & Dixon, 1951; Duff, Robinson & Smith, 1951; and others) that the 'degradation' of hyaluronic acid which occurs in pathological fluids decreases on hormonal therapy, but experimental data have been scarce.

Recently Jessar, Ganzell, & Ragan (1953) have reported that clinical response to intra-articular hydrocortisone in rheumatoid arthritis is reflected by an increase in viscosity and hyaluronic acid concentration (as measured by a turbidimetric method) in the fluids, whereas the change in total protein is inconsistent. In most of their cases there was, in addition, an increase in the degree of polymerization of the hyaluronic acid, as judged by changes in a polymerization factor calculated as log viscosity/hyaluronic acid concentration. This factor has been shown to be low in rheumatoid arthritis fluids as compared with normal fluids (Ragan & Meyer, 1949). It should be noted, however,

[1] The authors are indebted to Mrs. Barbro Wikman for valuable technical assistance. Financial support was obtained from King Gustaf V's 80 Years Foundation.

that the increase in the polymerization factor observed after hydrocortisone was comparatively slight, exceeding 20 per cent in only a few instances. Since the turbidimetric method of measuring hyaluronic acid seems to be influenced also by qualitative changes in the polysaccharide (Gibian, 1952), slight changes in a polymerization factor calculated on the basis of such methods should be judged with caution.

The aim of the present study has been to apply to this problem the methods of determining concentration and degree of polymerization of hyaluronic acid designed by Sundblad (1953, 1954). In the case of systemic hormonal therapy, no consistent effect on the qualitative polysaccharide changes of rheumatoid arthritis fluids could be demonstrated by these methods (Sundblad, 1953). The present investigation has been mainly confined to the action of intra-articular hydrocortisone, which has been shown by various workers (see *e. g.* Hollander, Brown, Jessar & Brown, 1951; Egelius, Hävermark & Jonsson, 1953) to exert a potent local effect in rheumatoid arthritis.

The action of hydrocortisone upon the content of hyaluronidase inhibitor in the fluids has been included. It is known (Håkanson & Luft, 1949; Schmidt & Faber, 1949; and others) that the non-specific hyaluronidase inhibitor of serum decreases following ACTH-administration in rheumatoid arthritis. In contrast to this it has recently been reported (Ekman, Thune & Truedsson, 1953) that in joint fluids of rheumatoid arthritis the amount of hyaluronidase inhibitor increases parallel to the increase in viscosity after repeated intra-articular injection of cortisone.

METHODS

Synovial fluid was obtained from the knee joints of patients with rheumatoid arthritis. Typical cases in an active stage and presenting slight or moderate degrees of effusion in one or both knee joints were selected for this study.

No attempt was made to empty the joint completely. In order to get an idea of the degree of effusion about 5 ml saline was injected into the joint cavity after withdrawal of the test sample in some of the cases. Mixture was achieved by repeatedly sucking and blowing with a syringe. Another sample was then withdrawn, and the viscosity of the two samples determined. The amount of fluid remaining in the joint after the initial puncture could then be calculated from the dilution of the mixed sample.[1]

The samples were stored at —20° C if not analysed immediately. The total protein was determined by a modified biuret method (Lehman, 1944). The hyaluronic acid concentration and intrinsic viscosity, $[\eta]$, were determined according to the method described by Sundblad (1953). The technical details of the viscosity determinations are given in the accompanying paper (Sundblad, 1954), where the method of measuring anomalous viscosity is also described. Values of $[\eta]$ given refer to viscosity determinations at a shearing stress of 17.0 dynes per cm². Anomalous viscosity is expressed as $\log \frac{[\eta]_7}{[\eta]_{17}}$, calculated from viscosity determinations on the same sample at 7 and 17 dynes per cm².

Determinations of both intrinsic viscosity and anomalous viscosity were ordinarily used in tracing changes in the degree of polymerization of the hyaluronic acid, except for some cases where no determinations of hyaluronic acid concentration were made.

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[1] For the calculation of the degree of dilution the formula given by Sundblad (1953) is employed, according to which the value of the expression $\frac{\eta_{sp}}{1 + 0.16\ \eta_{sp}}$ is directly proportional to the hyaluronic acid concentration. η_{sp} is the specific viscosity of the hyaluronic acid.

For the determination of hyaluronidase inhibitor vity a viscometric method of assaying hyaluro- ase activity (Sundblad, 1953) was used. De- dation rates were determined in the presence and ence of the inhibitor to be tested, and the per- tage decrease of the rate used as a measure inhibitor activity. 0.1 ml synovial fluid was abated for exactly one hour at 37° C with 1.1 purified testicular hyaluronidase dissolved in fer-NaCl of pH 7.0, containing 0.1 ml M $MgCl_2$ 100 ml. 2 ml hyaluronate[1] in buffer-NaCl was added, and 3 ml of the reaction mixture imme- ely transferred to the viscometer. Final con- ration of hyaluronic acid = 0.15 g per ml. By procedure the increment of η_{sp} due to the osity of the synovial fluid was negligible (below) with 0.1 ml fluid. The results obtained for m by this method are shown in Fig. 2. As be seen, a linear relationship is obtained by ting per cent inhibition against log concentra-

Duplicate determinations were performed ughout.

RESULTS

anges in hyaluronic acid

The synovial fluid was examined before d 2—3 days after intra-articular injection 50 mg hydrocortisone acetate (Hydro- tone Acetate, Merck) in 14 cases. Con- ning the findings of previous authors, the eficial local effect on the joints was in all es accompanied by a varying increase in d viscosity. In 11 of the cases the rise in cosity could be in part ascribed to an rease in hyaluronic acid concentration. e consistent change was, however, an in- ase in the degree of polymerization of the luronic acid as manifested by the values both intrinsic viscosity and anomalous cosity. In about half the cases the intrinsic cosity rose to essentially normal values ove about 40), as compared with the data given by Sundblad (1953). The results are summarized in Table I.

In 10 of the cases we attempted to measure the fluid volume as described above. The values before treatment ranged between 2—47 ml, average 20 ml, as compared with 0.5—7 ml, average 4 ml, 2—3 days after hydrocortisone. On an average the decrease in exudation was accordingly considerably greater than the increase noted in hyaluronic acid concentration (see Table I).

The duration of the effect of a single dose of hydrocortisone and the effect of repeated doses is shown in Fig. 1. In a few other cases tested by repeated aspiration 1—7 days after injection, the results were similar, with a maximum response after 2—4 days.

The results obtained in similar tests with intra-articular injection of some other compounds are given in Fig. 1 and Table II. Cortisone appeared to have a similar action on the hyaluronic acid as did hydrocortisone, although considerably less marked.

The clinical response was also slight in the tests with cortisone. Nor did butazolidin, which has little or no local effect in rheumatoid arthritis (Egelius & Nyström, 1954), influence the hyaluronic acid. A synthetic hyaluronidase inhibitor, polyphloretin phosphate[2] (Diczfalusy, Fernö, Fex, Högberg, Linderot & Rosenberg, 1953), was also tested, partly from theoretical considerations and partly since treatment with hyaluronidase inhibitors has been reported to be of clinical value in rheumatoid arthritis (Hahn, Thune & Truedsson, 1952). Under the conditions employed no effect on the hyaluronic acid could be demonstrated with

[1] Hyaluronate from umbilical cord, obtained ugh courtesy of Professor G. Blix, Uppsala.

[2] Obtained through the courtesy of Leo Ltd., Sweden.

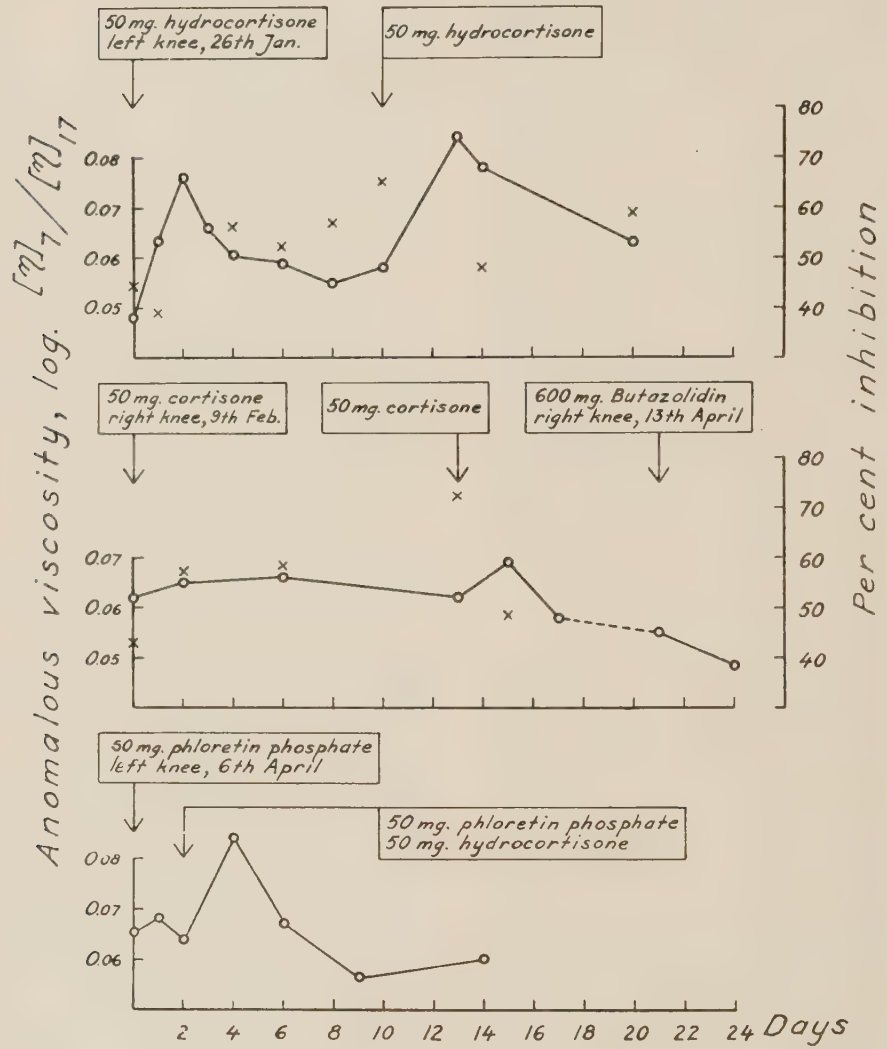

Fig. 1. Changes in the joint fluid of a case of rheumatoid arthritis following intra-articular injection of various agents.

-o-o- = changes in degree of polymerization of the hyaluronic acid as measured by the degree of anomalous viscosity.

x = hyaluronidase inhibitor activity, measured by the per cent inhibition, using 0.1 ml fluid. This test could not be performed in all instances due to shortage of test material. For comparison with the inhibitor level in the patient's serum, see Fig. 2.

able I. *Changes in concentration and degree of polymerization of the synovial aluronic acid in rheumatoid arthritis following intra-articular injection of 50 mg hydrocortisone.* (For the meaning of the statistical symbols t and P, see *e. g.* Fisher, 1948.)

Test	No. of patients	Average values before hydrocortisone	Changes 2—3 days after hydrocortisone			Average percentage changes
			Mean	t	P	
luronic acid concentration, per 100 ml	14	0.100	+0.054	3.29	< 0.01	+69
nsic viscosity, $[\eta]_{17}$	14	27.2	+9.1	5.40	< 0.001	+38[1])
malous viscosity, $\log \frac{[\eta]_7}{[\eta]_{17}}$	14	0.043	+0.028	7.45	< 0.001	+63[1])

[1] The changes in anomalous viscosity were often more marked than the changes in intrinsic viscosity. The probable explanation is that in such cases only a fraction of the hyaluronic acid is ghly polymerized. Even if this fraction were comparatively small, the degree of flow anomalies ay be pronounced, whereas the value of $[\eta]$ as a measure of the mean polymerization will be less affected (see Sundblad, 1954).

ble II. *Changes in degree of polymerion of the synovial hyaluronic acid, as asured by the anomalous viscosity, in umatoid arthritis following intra-articular injections of various agents.*

eatment	No. of patients	Percentage changes in $\log \frac{[\eta]_7}{[\eta]_{17}}$ (2-3 days after injection.)	
		Range	Mean
isone,) mg	5	+3 — +30	+16
azolidin, 00 mg	8	—24 — +15	0
oretin osphate —100mg	5	—14 — +5	—2

drug, nor did the inhibitor seem to inence the degree or duration of the changes yaluronic acid induced by hydrocortisone e Fig. 1). The dose of phloretin phoste used (50 mg) would, with regard to inhibitor activity, correspond to about 200 ml of the patient's serum, as calculated from *in vitro* tests.

Changes in hyaluronidase inhibitors and total protein

It can be seen from Fig. 1 that the changes in hyaluronic acid were not accompanied by any regular change in hyaluronidase inhibitor activity in the fluids. Also, the total protein varied irregularly in this case.

A comparison with the data of Fig. 2 shows further that the inhibitor activity is low, the level in the serum being on an average about 5 times that in the fluids. The effect of the addition of a drop of toluene to the test sample before assaying the inhibitor activity is also illustrated in Fig. 2. Toluene has been reported to abolish the inhibitor action of serum (Adner, 1948),

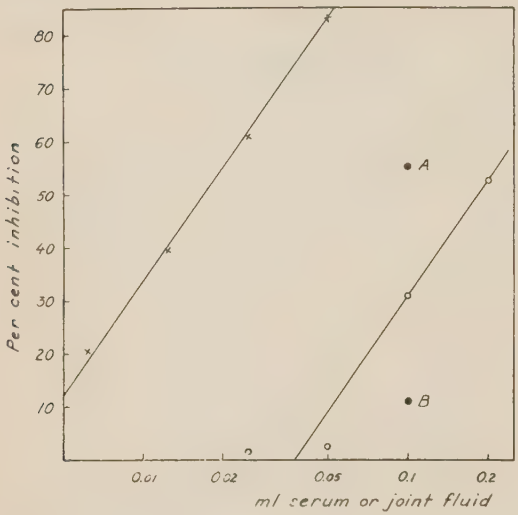

Fig. 2. Hyaluronidase inhibitor activity in serum and joint fluid of a case of rheumatoid arthritis. Same case as in Fig. 1. Duplicate determinations of inhibitor activity with and without addition of a drop of toluene to the test sample were performed.
x-x-x- = serum; no toluene.
-o-o-o- = serum; toluene added.
A = mean value of 13 joint fluids; no toluene.
B = mean value of 11 joint fluids; toluene added.

which could be confirmed except for the highest serum concentrations tested. In contrast to this, the *in vitro* inhibiting effect of heparin and phloretin phosphate on testicular hyaluronidase was found to be uninfluenced by toluene. In the joint fluids too the inhibitor activity was almost completely abolished in the presence of toluene, which seems to support the idea that the inhibitor in joint fluid is similar to that in serum.

The results of estimations of inhibitor activity and total protein in some additional cases are given in Table III.

A slight but significant decrease in inhibitor activity occurred in the fluids following administration of hydrocortisone. The changes in total protein were insignificant. The hydrocortisone could not be shown to have any effect on the inhibitor activity of the fluids *in vitro*.

Table III. *Changes in hyaluronidase inhibitor activity and total protein in joint fluids in rheumatoid arthritis following intra-articular injection of 25—50 mg hydrocortisone.*

Test	No. of patients	Average values before hydrocortisone	Changes 2 days after hydrocortisone		
			Mean	t	P
Hyaluronidase inhibitors, per cent inhibition with 0.1 ml fluid.	9	55.2	—10.0	6.95	<0.001
Total protein, g per 100 ml	10	4.11	—0.41	2.12	< 0.1

DISCUSSION

It was found that the clinical response to local treatment of rheumatoid arthritis with hydrocortisone was regularly accompanied by an often complete restoration to normal of the qualitative hyaluronic acid changes occurring in the joint fluids in this disease. The change in the degree of polymerization of the polysaccharide was manifested by a marked increase in both intrinsic viscosity and degree of flow anomalies.

Although the degree of clinical improvement is difficult to assess accurately, we had the impression that it closely paralleled the degree of biochemical change. Nor was there any appreciable influence on the hyaluronic

l in cases where the clinical response was ht or absent, as in the experiments with tisone and butazolidin.

he quantitative changes in the fluids uced by the hormone were less consistent. re was, as a rule, an increase in concen- ion of hyaluronic acid with slight changes otal protein. If, however, the change in d volume is taken into consideration, the inished release of fluid and proteins into joint cavity was usually paralleled by a rease in the total hyaluronic acid content he fluids, which is often high in rheuma- arthritis (see Sundblad, 1953).

he *qualitative* change in the hyaluronic could hardly be related to changes in neability of the synovial barrier induced the hormone. The likely explanation of effect would be that the hormone acts er by inhibiting hyaluronidase or by in- ncing the synthesis of the hyaluronic in the synovial connective tissue.

he former alternative is not supported experimental data. Hyaluronidase has not n demonstrated in synovial fluids con- ing depolymerized hyaluronic acid. The sent studies on hyaluronidase inhibitors e, on the contrary, shown a significant rease in inhibitor activity in the fluids r the local use of hydrocortisone. Simi- y, the inhibitor activity of serum is known lecrease on systemic hormonal treatment rheumatoid arthritis.

n fact the proof of a connexion between copolysaccharide metabolism and hyalur- dase inhibitors seems still to be lacking. ce no appreciable inhibitor activity is nd in normal joint fluid (Wattenberg & k, 1949) it would appear that the presence he rheumatoid fluids of small amounts of inhibitors of presumably the same type as in serum is the result of exudation into the joint cavity. The decrease in inhibitor activity observed after hydrocortisone might then simply be related to the decrease in exudation.

It would seem reasonable to ascribe the hyaluronic acid changes observed after hydrocortisone to an action of the hormone on the synovial connective tissue, where the polysaccharide is presumably synthesized. As a result of this effect the depolymerized hyaluronic acid in the fluids is replaced after 2—4 days by a nearly normal product.

This action on hyaluronic acid synthesis may well be secondary to the non-specific anti-inflammatory action of the hydrocortisone, and unrelated to the beneficial clinical effect. The possibility that the influence on mucopolysaccharide metabolism may be an essential feature of the hormonal action in rheumatoid arthritis does, however, warrant further study.

SUMMARY

Clinical response to intra-articular treatment of rheumatoid arthritis with hydrocortisone was always reflected by chemical changes in the joint fluid.

There was usually an increase in hyaluronic acid concentration, but little or no change in the total protein.

The consistent change was an increase in the degree of polymerization of the hyaluronic acid, as manifested by a rise in both intrinsic viscosity and degree of anomalous viscosity. The maximum response occurred 2—4 days after injection with roughly normal levels of intrinsic viscosity in about half the cases.

The change in the hyaluronic acid could hardly be ascribed to inhibited depolymerization, since there was a slight but significant decrease in hyaluronidase inhibitor activity in the fluids.

The hormone probably acts by influencing the synthesis of hyaluronic acid in the synovial tissue either directly or indirectly by its anti-inflammatory action.

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A MODIFIED METHOD FOR THE DETERMINATION OF PEPSINOGEN IN URINE (UROPEPSIN)

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Quantitative estimation of urinary pepogen excretion — uropepsin (UP) — received renewed attention since Spiro, ifenstein & Gray (1953) demonstrated a rked increase in UP-output, following ministration of ACTH. This observation nted towards UP as a useful indicator gastric secretory activity, especially where monal regulation of this activity is conned. Mirsky, Kaplan & Broh-Kahn (1950) re able to demonstrate an increase in UPmination during periods of "stress and xiety" in individuals undergoing psychorapy. Gray, Ramsey, Reifenstein & Ben (1953) made extensive use of UPerminations in a study of hormonal facs in peptic ulcer.

The present paper describes a modified thod for the assay of UP, with a brief cussion of some factors that bear upon h determinations.

EARLIER METHODS

Brücke (1861), who first discovered UP, rked with fibrin and coagulated eggite. Other early work was done with ütznerʼs fibrin method (1887). The use substrates in solution was introduced by ms (ricin) (1907) and Fuld & Levison lestin) (1907). Gottlieb (1925) developed a rather satisfactory method from Ege's modification of Fuld's edestin method.

Bucher (1947) adapted the hemoglobin method of Anson (1939) to UP. Further modifications were introduced by Mirsky, Block, Osher & Broh-Kahn (1948) and by Fawcett (1951).

While the above-mentioned methods depend upon estimation of protease activity, Sylvest (1949) has given a method for the determination of renninogen activity in urine (urorennin); West, Ellis & Scott (1952) and Goodman, Sandoval & Halsted (1952) have worked with this method, introducing further modifications.

THE PRESENT METHOD

is based upon Anson's hemoglobin method; it differs from Bucher's and Mirsky's modifications mainly by:

1) the introduction of dialysis of urine specimens prior to assay of proteolytic activity;

2) by applying ultraviolet photometry to the estimation of aromatic amino acids liberated during proteolysis. This latter principle was apparently first mentioned by Kunitz (1947); it is discussed in detail by Pantlitschko, Kaiser & Andres (1952).

Substrate

Hemoglobin is prepared from ox-blood according to Anson (1939); it is convenient to store it in the lyophilized state. The content of protein is

estimated by Anson's drying and weighing method. Substrate is prepared daily from a stock solution of 5 per cent (with 2.5 per cent merthiolate 1 : 1000 added as preservative). To 15 ml of stock solution add 6.5 ml 0.5 N HCl and fill up the volume to 50 ml with distilled water. This substrate has a pH of 1.4, contains 1.5 per cent hemoglobin.

Dialysis

15 ml samples of urine are dialyzed against distilled water in pieces of cellophane tubing approximately 1.5 cm in diameter, closed by knots tied at both ends. In each cellophane bag thus formed, a glass bead is enclosed. Dialysis is carried out in a glass cylinder, closed with a ground-glass lid and a clamp. A 6 L cylinder is suitable for the simultaneous dialysis of 10—12 samples. The cellophane bags are suspended in the cylinder on a frame consisting of two U-shaped glass rods, which are held together with pieces of rubber tubing; the loose ends of the bags are clasped between the two parts of the frame. For stirring, the cylinder is rotated in the horizontal position on two rubber-covered rollers, powered by an electric motor. The outer fluid is renewed after ½ hour, and after 4 hours' dialysis the cellophane bags are emptied by gently puncturing them with a pointed glass rod. Through a funnel the sample is transferred quantitatively to a 20 ml volumetric flask. One knot is cut off the tube, which is then washed with a few ml of water into the flask; the volume is made up to 20 ml with water.

Procedure

Into one branch of a Λ -shaped vessel are pipetted 2 ml of substrate, into the other branch 1 ml of dialyzed urine, and the vessel is placed in the water-bath at 37°. After 10 minutes the reaction is started by tilting the vessel to and fro to obtain thorough mixing of substrate and urine, and the reaction mixture is finally left in one branch of the vessel during incubation. After exactly 30 minutes the reaction is stopped by the rapid addition of 10 ml of 0.3 N trichloracetic acid — conveniently from a Krogh syringe pipette.

Blanks are prepared in 50 ml Erlenmeyer flasks by adding, in the order mentioned: 1 ml of urine, 10 ml of trichloracetic acid, and 2 ml of substrate.

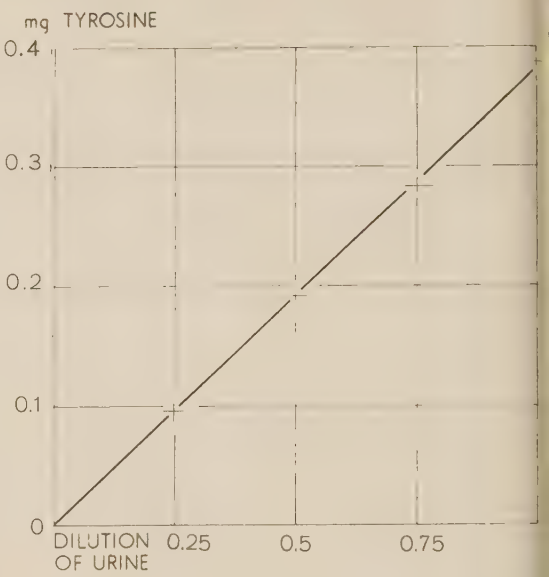


Fig. 1. Relation between concentration of uropepsin and amount of aromatic amino acids liberated. Time: 30 min. Tp. 37°. pH 1.6.

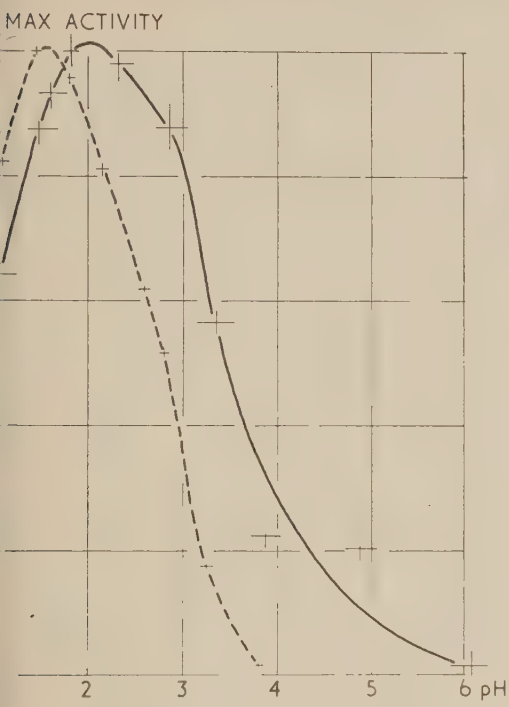
Blanks and samples are filtered through paper (Whatman No. 50) into bottles, which are left stoppered until photometry.

The amount of aromatic amino acids in the trichloracetic acid filtrate is estimated by reading the extinction coefficients against 0.3 N trichloracetic acid in 10 mm quartz cuvettes at 2750 Å, with a slit width of 0.4 mm).[1]

Calculation

The amount of aromatic amino acids liberated by 1 ml of urine during proteolysis is given as: (mg tyrosine in sample) ÷ (mg tyrosine in blank) × 1.33 — (to correct for dilution of urine following dialysis). Fig. 1 demonstrates that a linear relationship exists between UP-concentration and amount of aromatic amino acids in the trichloracetic acid filtrate.

[1] A "UNICAM" quartz spectrophotometer was used.



2. Relation between pH of the digestion mix- and proteolytic activity (expressed as per cent maximum activity found). Time: 30 min. 37°. + - - - - +: crystalline pepsin "Armour". —— +: human gastric juice, diluted 1:400.

Calibration

Dissolve 50 mg l-tyrosine in 100 ml N/10 HCl; exact amount of tyrosine in solution is deter- ed by the Kjeldahl procedure (tyrosine con- s 7.731 per cent N). A suitable dilution series repared as follows: to 10 ml of 0.3 N trichlor- ic acid add 0.5—1.0—1.5—2.0 ml of tyrosine tion; make up the volume to 13 ml with N/10 l. When extinction coefficients are plotted nst mg tyrosine, a straight line is obtained ch can be used to convert photometric readings mg tyrosine present in the reaction mixtures. lesired, a conversion factor can be calculated n the calibration data and made to include the ion factor.

Table I. *Effect of dialysis on blank value. Figures refer to photometric readings.*

	No dialysis	Dialysis
Sample	1.13	0.199
Blank	1.04	0.101
Difference	0.09	0.098
Difference per cent of blank	8.7	97.0

COMMENT

Dialysis of samples serves 1) to eliminate the buffering capacity of urine; 2) to reduce blank values.

1) Addition of the untreated urine to the substrate influences pH of the digestion mixture to a considerable and varying degree. The desirability of carrying out estimations of enzyme activity at a well-defined pH is obvious; while Bucher's method disregards this, Mirsky *et al.* found it necessary to titrate urine specimens with HCl to the pH desired. When dialyzed urine is used, there is no shift in pH beyond that resulting from dilution of substrate. This is constant and can be determined beforehand. The buffering capacity of the acid hemoglobin suffices to maintain pH at a constant level during digestion. With the present method, deviations in pH did not exceed the experimental error of the pH-meter used.

2) With undialyzed urine, blank values exceed by several times the increase in ultraviolet extinction obtained during proteolysis, unless extremely long digestion times are employed. The same is true when the Folin-Ciocalteau reagent is used to determine aromatic amino acids, as in Anson's method. This affects the accuracy of the method un-

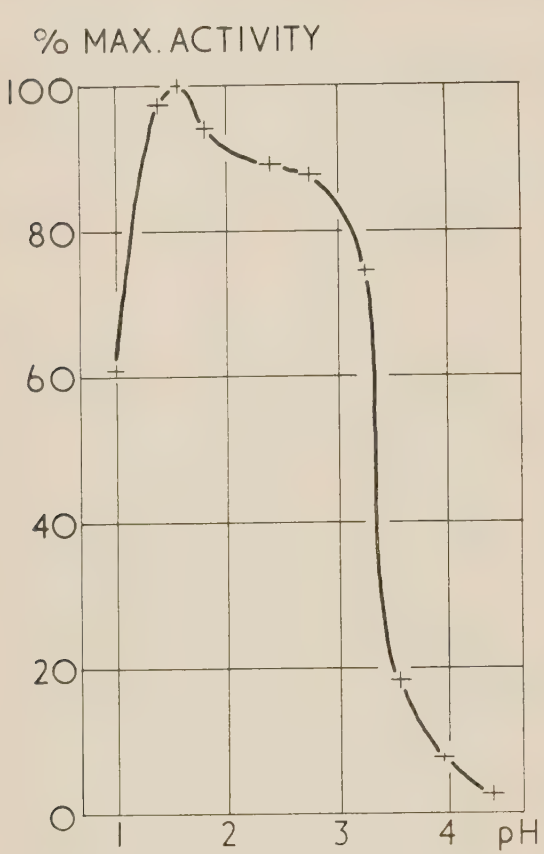


Fig. 3 a.

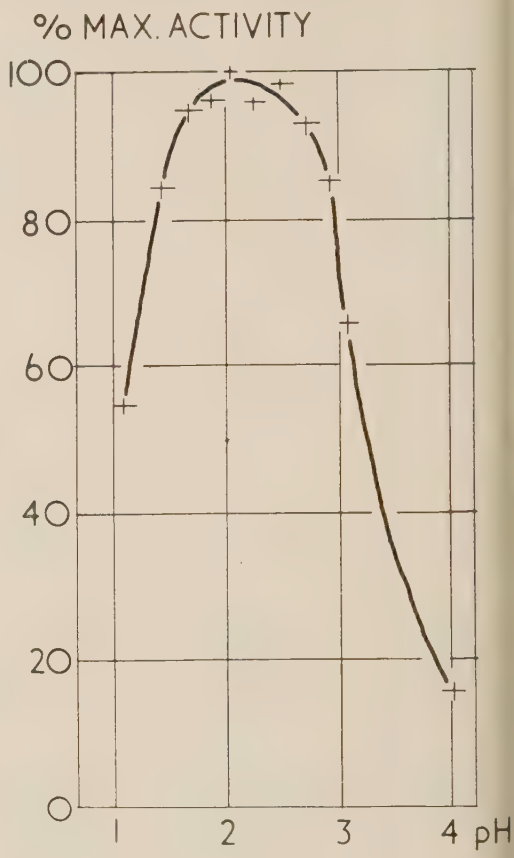


Fig. 3 b.

Fig. 3. Relation between pH of the digestion mixture and proteolytic activity in two different specimens of dialyzed urine. Time: 30 min. Tp. 37°.

favorably, especially where low degrees of proteolysis are concerned. With dialysis as described above, blank readings are reduced to the same order of magnitude as the increase obtained during proteolysis with a urine specimen of average digestive capacity (Table I). The reduction in blank value during 4 hours' dialysis is about 90 per cent of the initial level. Peptic activity is not influenced by dialysis (Mirsky *et al.* 1948).

pH of the digestion mixture

Fig. 2 demonstrates proteolytic activity o 1) crystalline pepsin (Armour) and 2) hista mine stimulated gastric juice, using the pre sent method. Whereas Armour pepsin ex hibits a definite optimum at pH 1.5—1.6 gastric juice shows maximum activity aroun pH 2, and still about 60 per cent activit at pH 3.5 where crystalline pepsin is prac tically inactive.

reudenberg (1940) has demonstrated the sence in gastric juice of a gastric kathep- and Buchs (1947), using a nephelometric hod, postulates the presence in crystalline sin as well as in gastric juice, of two inct components: one pepsin-like with its imum at pH 2.2, and another kathepsin- with an optimum at pH 3.3. Though results of Buchs have not been confirmed h other methods for determination of teolytic activity, some uncertainty re- ns as to the exact nature of the gastric tease and its relationship to crystalline sin of Northrop's type.

he curves obtained with specimens of yzed urine, two of which are given in . 3, show a close resemblance to the one ained with gastric juice, with slight varia- s, however, from specimen to specimen. se variations may be due to an influence substances other than proteases present in ne. It is not unlikely, however, that small varying amounts of kathepsin-like en- es from sources other than the gastric ds are eliminated through the kidneys, these may influence the activity curves urine.

or the present purpose, a pH of 1.6 is sen for the determination of UP, in er to avoid the possible influences of hepsin-like proteases.

Incubation time

ig. 4 demonstrates the relationship be- en incubation time and the amount of sine-like substances liberated. This rela- ship is rectilinear well beyond the 30 utes used in the present method.

ucher (1947) used 60 minutes, whereas sky *et al.* (1948) found that this could

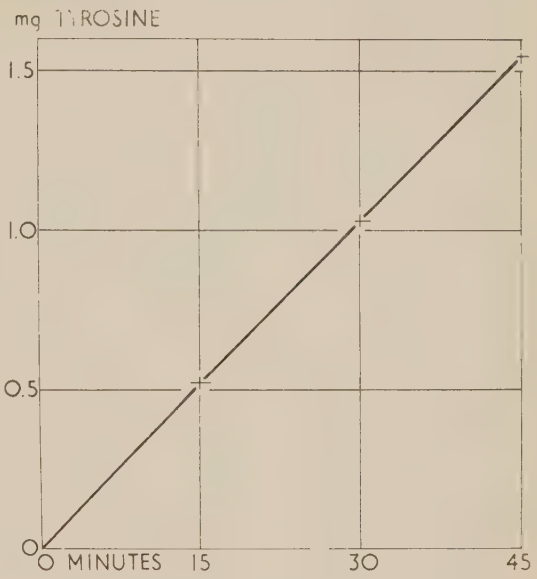


Fig. 4. Relation between incubation time and amount of aromatic amino acids liberated. Dialyzed urine. Tp. 37°. pH 1.6.

be shortened to 10 minutes in most cases; when low activities were encountered, the assay was repeated with a 30 minutes' digestion time; in a later communication (1950) they have used a longer incubation time — (and at the same time a higher dilution of urine).

With the present method, an incubation time of 30 minutes was found to yield a suitable range of "tyrosine values" for the range of UP activities met with, *i. e.* permitting low activities to be well quantitated, yet keeping even very high activities well within the rectilinear portion of the digestion curve.

Units

The UP activity of 1 ml of urine is expressed as mg tyrosine liberated from the substrate under the experimental conditions

described. Most other authors do the same, and as methods vary, so do "units".

It would obviously be desirable to be able to express UP-activities as actual amounts of enzyme. Sylvest (1949) and West, Ellis & Scott (1952) use commercial pepsin preparations for their urorennin determinations. An actual standard pepsin is, however, not available. Also, the considerations given above, concerning the dissimilarities between crystalline pepsin and the gastric protease, would seem to make the use of a pepsin standard inadvisable; but an approximate indication of the unit in terms of pepsin will serve purposes of comparison between different methods.

With the present method, 1 mg tyrosine equivalates the activity of approximately 10 gamma crystalline pepsin Armour.

UP excretion

UP excretion is, as a rule, given as mean excretion per hour in a 24-hour period. The 24-hour diuresis is collected and measured, and a sample taken for analysis. Urine should be stored cold (4—5°), as there is significant loss of activity at room temperature. The rate of decrease in activity is greater with specimens of high activity, as one would expect when it is assumed that this process is autocatalytic. There is no advantage in adding toluene as a preservative.

Mirsky *et al.* (1948) found that variations in UP excretion rate, as determined separately for the sleeping and the waking period of the subjects examined, were not statistically significant. Nor could they demonstrate significant differences in this respect between consecutive periods of the same day. On the other hand, Goodman *et al.* (1952) — using the urorennin method — found considerable variations between consecutive 4-hour periods. West *et al.* (1952) found "morning values consistently higher than afternoon and night values".

In 11 hospitalized individuals without gastrointestinal disease, the author found 8 (representing 33 consecutive days and 35 consecutive nights) excreting an average of 32.6 per cent more UP by day than by night; range: 18.5 per cent—89.4 per cent. Whereas 3 (representing 12 days and 13 nights) had night excretions exceeding day excretions by 17.0, 33.3, and 79.0 per cent respectively. — Obviously the excretion pattern of UP needs further study.

Accuracy

The information available concerning experimental accuracy and sources of error in UP determinations is scarce (Mirsky *et al.* (1948); Goodman *et al.* (1952).

Table II gives the result of 10 assays performed on the same specimen of dialyzed urine.

An estimate of the working accuracy of the method, calculated from 74 routine double determinations (Hald, 1948) is tabulated in Table III. This calculation comprises all steps of the analytical procedure, including dialysis.

Additional sources of error in estimation of UP output arise from measurement of diuresis: inadvertent loss of part of the diuresis, incomplete emptying of the bladder, error in timing and in volumetry. Under clinical conditions, error arising from these sources may be considerable, but it can not be evaluated in any simple manner.

able II. *10 assays performed on one ecimen of dialyzed urine. Figures refer to photometric readings.*

No.	Blank	Sample	Sample — blank
1	.101	.199	.098
2	.104	.203	.099
3	.106	.200	.094
4	.101	.199	.098
5	.105	.197	.092
6	.103	.196	.093
7	.098	.198	.100
8	.096	.202	.106
9	.101	.201	.100
10	.105	.208	.103
ean.....	.102	.200	.098
). of mean	.0032	.0035	.0044

able III. *Estimation of experimental ror in 74 routine double determinations.*

	Extinction values	Tyrosine mg
nge	.016 — .449	.035 — .992
erage.....	.160	.354
riance	.000113	
).	.0106	(.023)

Degrees of freedom: 74.

SUMMARY

A method for the quantitative determina-n of urinary pepsinogen (uropepsin) is scribed, in which hemoglobin is used as a bstrate; the degree of proteolysis is ex-essed as amount of aromatic amino acids erated from the substrate. This is deter-ned by reading the extinction coefficient the trichloracetic acid filtrate at 2750 Å. It is shown that dialysis of urine speci-ens prior to assay of proteolytic activity substantially reduces blank values which e otherwise very high, and 2) simplifies justment of the digestion mixture to the l desired.

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A METHOD FOR DETERMINATION OF ACETONE IN EXPIRED AIR — WITH SPECIAL REFERENCE TO DIABETIC KETOSIS[1]

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Clinically it is well known that expired air n comatous diabetics may have a very racteristic smell. About one hundred rs ago Lerch suggested that this might due to acetone. Rupstein (1874) was the t who made expired air from a diabetic ble through cold water and showed — by ns of Lieben's iodoform reaction — that robably contained acetone.

t is rather strange that analytical methods tracing acetone in expired air have not ed ground although several authors mpted to materialize this idea (Scott-lson, 1911; Briggs & Shaffer, 1921; Her-n Nielsen, 1943). The explanation is bably that no sufficiently sensitive and ple method has been introduced. Provided h practically applicable method were avail-e, a determination of acetone in expired would certainly have advantages over analysis of urine for ketones.

f the patient is unconscious, as in diabetic na, the requirement of a urine analysis olies catheterization which — besides in-ving the danger of infection of the urinary ct — may also be difficult or even im-sible to perform, *e. g.* in infants and ients suffering from hypertrophy of the prostate or urethral stricture. In cases of shock with a reduced glomerular filtration the urinary concentration of ketones may not rise to amounts which can be determined by routine methods although the ketones in the blood are markedly increased. Finally, the bladder may contain no urine, either because it has been emptied shortly before or because no urine is being produced.

If a sufficiently simple analysis were available for tracing acetone in expired air it would prove a much faster and simpler method than the determination of acetone in urine, and it would certainly give a more reliable picture of the patient's acetonemia. Repeated tests might thus be made without any inconvenience to the patient and the acetonemia might be more closely followed; this is not possible if only urine analyses are performed and, particularly in the treatment of comatous diabetics, frequent analyses must be considered highly important.

EARLIER METHODS FOR THE DETERMINATION OF KETONES

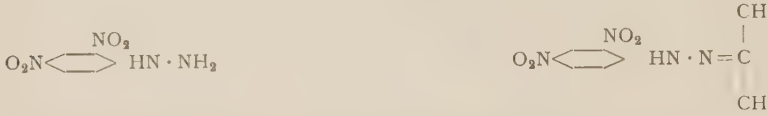
Tests of sensitivity of methods

From among the numerous methods for the determination of ketones only the most important are recorded here.

[1] Supported by a grant from King Christian X d.

We have tested these methods on aqueous, freshly prepared acetone solutions.[1] Table I gives the sensitivity[2] in mg of acetone per 100 ml as well as in μg of acetone in 2 ml water, *viz.* the volume adopted in our method.

Since Curtius & Dedichen (1894) described the compounds



2,4-dinitrophenylhydrazine — Acetone-2,4-dinitrophenylhydrazone (= isopropyliden-2,4-dinitrophenylhydrazine)

$C_6H_6O_4N_4$ — $C_9H_{10}O_4N_4$.

M = 198.14, melting point 194° C. Red, crystalline.

M = 254.20, melting point 128° C. Yellow, crystalline.

the hydrazine (hereinafter called DNPH) has proved to be superior to all other carbonyl group reagents, because hydrazones develop rapidly (Friedemann & Haugen, 1943) and crystallize easily (Allen, 1930).

The hydrazine is insoluble in water and in carbon tetrachloride, and is only slightly soluble in ethanol; the hydrazone is almost insoluble in water, but is soluble in carbon tetrachloride and ethanol.

Previously, ethanol, sulphuric acid, and hydrochloric acid were used as solvents for DNPH — according to the requirements of the reagents. DNPH is easily dissolved in sulphuric acid, much less so in hydrochloric acid (about 0.42 per cent at room temperature, Iddles & Jackson, 1934). Saturated ethanol solutions range about 1 per cent, but during the reaction with the carbonyl compound the hydrazone thus developed is prone to "coat" the hydrazine and prevent any further development of hydrazone (Brady, 1931).

For the past twenty years numerous qualitative as well as quantitative methods have been recorded by which DNPH has been applied for tracing carbonyl compounds in air, blood, and urine. Greenberg & Lester's method (1944) should be particularly emphasized: 0.1 per cent DNPH in 2 N of hydrochloric acid; extraction by carbon tetrachloride; washing with 0.5 N of sodium hydroxide and water; subsequent colorimetric reading. Herman Nielsen stated in 1943 how acetone might be traced in expired air by DNPH "if only the patient breathes into a test tube containing the reagent". According to our experience this requires, however, a pronounced acetonemia. Scot-Wilson (1911) suggested his reagent as a medium for the determination of acetone in expired air; and his method was recommended by Briggs & Shaffer (1921). So far none of the above mentioned methods seems to have gained ground.

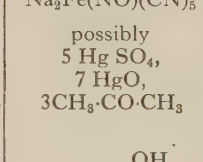
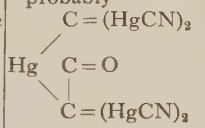
AUTHORS' METHOD FOR THE DETERMINATION OF ACETONE IN EXPIRED AIR

Principle and technique

For a period of 1—2 minutes the patient's expired air is led through 2 ml of a solution of 0.2 per cent dinitrophenylhydrazine in 2 N hydrochloric acid. Acetone yields acetone-dinitrophenylhydrazone which precipitates in scanty, yellow, delicate crystals, provided the expired air contains large amounts of acetone. Otherwise 2 ml of carbon tetrachloride is added, and the mixture shaken vigorously for 10—15 seconds. The appearance of a yellow color in the bottom layer (the carbon tetrachloride layer) indicates acetone since carbon tetrachloride extracts acetone-dinitrophenylhydrazone but not DNPH. If only a pale yellow

[1] Analytical reagent (Merck).

[2] In this paper "sensitivity" should be interpreted as: smallest amount of acetone to yield determinable positive reactions by the applied procedure.

Authors	Reagents	Final compounds in the reaction "Indicator"	Aspect of "Indicator"	Sources of error	Recorded (in urine or aqueous solution)		Tested (in aqueous solution)	
					μg per 2 ml	mg in 100 ml	μg per 2 ml	mg in 100 ml
Lieben (1870)	Alkaline iodine solution	CHJ_3	Yellow crystalline precipitate (smell of saffron)	All methyl-ketones and alcohols oxidized to them (spec. ethanol,) acetaldehyde	10 (within 3 min.)	0.5	10	0.5
Legal (1883) Borisch (1907)	sodiumnitro-prusside, sodium-hydroxide solution, acetic acid solution	possibly condensation product between $CH_3 \cdot CO-$ and $Na_2Fe(NO)(CN)_5$	red coloring	aceto acetic acid, acetaldehyde	2000	100	100	5
Denigès (1898) Oppenheimer (1899)	sulphuric acid solution + mercuric oxide (contains mercuric sulphate). Heating.	possibly $5\ HgSO_4$, $7\ HgO$, $3CH_3 \cdot CO \cdot CH_3$	white opalescence white precipitate	aceto acetic acid	40 7	2 0.35	100	5
Fabinyi (1900) Frommer (1905)	solid potassium hydroxide, salicylic aldehyde. Heating (70°C)		interfacial dark red colour	aceto acetic acid (acetaldehyde)	1	0.05	8	0.4
Marsh & Struthers (1905) Scott-Wilson (1911)	a solution of mercuric cyanide + argentic nitrate + sodium hydroxide	probably 	white opalescence white precipitate	all methyl ketones	"Very sensitive"		2	0.1
Galat. Ingram (1944) Barac & Colette (1949) Lestradet (1951) Page (1953)	pulvers of sodium nitroprusside + anhydrous sodium carbonate + sulphate of ammonia. (Galat: 1:220:275)	see Legal's reaction	red-violet coloring violet coloring	aceto acetic acid, acetaldehyde	as Legal 200 200 80—100	 10 10 4—8	 100 (best readability as to blanks in *Galat's* modification)	 5

color appears, a further 2 ml of 5 N sodium hydroxide may be added and the mixture shaken. The reaction is positive if the pale yellow coloring persists in the bottom layer; if it becomes colorless the reaction is negative. With some experience blanks should not be necessary except when the reaction is performed in electric light which lends the blank a yellow tint. Artificial day light is of no advantage. The concentrations of the reagents have been chosen so as to obviate the need of exact measurements, provided the ratio of the liquid quantities approximates 1:1:1.

The expired air from the patient is led to the DNPH solution through a glass tube of the type used in most laboratories for van Slyke & Cullen's urease method to determinate the blood urea. The tube ends in a bulb with five holes to reduce the size of the bubbles and increase the absorption of acetone. To the other end is fitted a rubber tubing with an ordinary, replaceable, glass-mouthpiece; all parts of the apparatus should be rinsed in water between tests.

If the patient is unconscious it is recommended that the mouthpiece be inserted between his lips and, while supporting his chin with one's thumb, compressing his nasal wings with the fore- and middle-finger. The patient's expired air is thereby led through the mouthpiece. (Fig. 1.) By releasing the pressure on the nasal wings and the chin the inspiration is allowed to proceed freely, a factor which prevents any aspiration of the reagent; this is further prevented if the rubber tubing be compressed between the thumb and forefinger of the other hand during inspiration while the test tube is held firmly in the hollow of the hand with the middle finger, ring finger, and little finger. *In infants* it is advisable to replace the mouthpiece by an olive-shaped bulb wedged into one nostril while the other nostril is compressed. The examination may prove impracticable to very excited patients.

Both for practical and mnemonic reasons the figure "2" has been chosen for most quantities and concentrations. If no reagent is to be wasted during vigorous bubbling, 2 ml of liquid in an ordinary test tube proves to be the largest quantity practicable. Smaller quantities reduce the absorption of acetone. The final volume of six ml proves the largest quantity manageable if the tube is shaken with no stoppering.

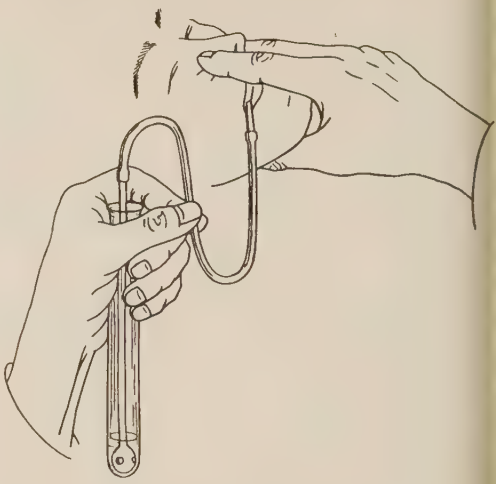


Fig. 1. Authors' technique for obtaining sample of expiratory air.

The duration of the bubbling process must to some extent be adapted to the efficiency with which the expired air from the patient passes through the tube. The volume of about 20 expirations must be considered adequate and is usually attained within 1—2 minutes.

If continued for any longer period, thus allowing larger amounts of air to pass through the reagent, greater quantities of acetone will also be absorbed — all other conditions being equal. Even minute percentages of acetone in the expired air may thus be traced. In our opinion the bubbling-through of 20 expirations is adequate to give the reaction a suitable clinical sensitivity; this procedure gives no positive reaction in normal subjects.

The reagents

0.2 per cent 2,4-dinitrophenylhydrazine in 2 N hydrochloric acid is produced by heating — while stirring — the weighed-out DNPH [1] with 2 N hydrochloric acid (a little less than the quantity required) up to 60—70° C (the glass is warm but not hot to the touch). In a few minutes the solution will appear clear orange-yellow, whereupon the

[1] Analytical Analar reagent with maximal impurity (as sulphate) 0.05 per cent.

inder of the hydrochloric acid is added. The nt should be stored in a refrigerator where it ins clear for months (so far up to 18 months). w red crystals may slowly precipitate at room erature but they do not prevent the use of upernatant without filtration; even a notice- decrease in the DNPH concentration fails to nce the sensitivity, provided the initial con- ation was about 0.2 per cent.

norough examinations have been made to tain whether or not the sensitivity of the od is enhanced by: *variations of the types of* (*e. g.* sulphuric acid, hydrochloric acid), *the ntration of the acids* (1—10 N) or *the DNPH ntration* (0.1—5 per cent). This was not the although the DNPH dissolves more readily ncentrated sulphuric acid than in the 2 N chloric acid used. High concentrations of chloric acid reduce the sensitivity to some t and are unpleasant to handle. Reduced con- ations of acids and DNPH curtail the sensi- to some extent, and decreasing concentrations ids reduce the solubility of DNPH thus im- g the stability of the solution.

e carbon tetrachloride should be pure analytical nt since a few commercial reagents yield pro- ced yellow blanks; it is stored in dark green s. Shaking with DNPH solution gives an ble emulsion which rapidly separates if stand- The carbon tetrachloride which forms the layer may show a yellow tint — particularly ent in electric light because of a dissolution inute quantities of DNPH. Only trifling nts of acetone may be confused with this blank . Extraction of the acetone hydrazone is, for actical purposes, quantitative in cases of small nts of acetone, as the aqueous phase scarcely any reaction when separated and shaken with onal carbon tetrachloride.

her agents for extraction have been examined, ethyl ether, ethyl acetate, chloroform, benzol, e, and benzene; but, as expected, they all ve DNPH to such an extent as to make the s a bright yellow.

e influence on blanks and sensitivities of *the of the hydroxide* (sodium and potassium xide), *the concentrations of the hydroxide* 0 N), and *the quantities of the hydroxide* (1—8 ml) has been thoroughly investigated but with the primary proviso that requirements to exactness of quantities should not be too severe *i. e.* with allowance for deviations between 1.0—3.0 ml). Potassium hydroxide was found to impair the coloring of the acetone-hydrazone more than equinormal sodium hydroxide; the concentration of sodium hydroxide should be about 5 N (or about 20 per cent w/v) to yield an almost colorless blank. Lower concentrations either restricted the variations allowed for in the ratio of the quantities of the reagents or resulted in yellow blanks. Increasing hydroxide concentrations bleached the hydrazone coloring in the carbon tetrachloride layer. The hydrochloric supernatant turns a brownish hue on addition of hydroxide; further additions — after neutralization of the acid — make it almost black, opaque, and warm; and still further additions yield a yellow-brown or red coloring. This breaking down of the DNPH results in various condensation and decomposition products like ammonia and nitrogen. Nothing much is known about the reaction mechanism by which the hydroxide influences the pale yellow blank; the quantity and concentration of the hydroxide is here empirically fixed. The trifling amount of DNPH dissolved in the carbon tetrachloride may be destroyed as mentioned above, whereas the hydrazone fails to be influenced. The alkaline supernatant, however, will extract a small amount of hydrazone from the carbon tetrachloride layer; the loss is partly compensated by an increase in the intensity of the hydrazone coloring in alkaline media (Greenberg & Lester, 1944).

Shaking with carbon tetrachloride, and hydroxide, if any, must be vigorous for 10—15 seconds to ensure a well dispersed emulsion and thus a satisfactory extraction. Provided the proper quantities of the liquids are fairly maintained — *i. e.* a total of about 6 ml — no stoppering of the test tube is required.

SENSITIVITY OF METHOD. ESTIMATE OF ACETONE CONTENT

The sensitivity was tested on 2 ml of the DNPH reagent to which 0.1 ml of various concentrations of freshly prepared, aqueous solutions of acetone were added (a fresh

ampule with analytical reagent (Merck) was used for each test).

The smallest quantity determinable was 8 μg corresponding to 0.4 mg per 100 ml. Careful scrutiny and blanks increase the sensitivity to about 4—6 μg. The following procedure makes it possible to get an estimate of the concentration and a rough quantitative idea of the amount of bound acetone:

More than 1000 μg (= 1 mg): A voluminous, yellow, flocculating precipitate immediately appears in the DNPH reagent; the liquid proper becomes almost colorless because the DNPH has been quantitatively converted into crystallized hydrazone. (The DNPH solution being 0.2 per cent and the molecular weight of DNPH being 198.14, the molar concentration will amount to about $10^{\div 2}$ M; thus there will be $2 \times 10^{\div 5}$ gram-moles in 2 ml. The molecular weight of acetone is 58.08, *i. e.* $2 \times 10^{\div 5}$ gram-moles equal 1.16 mg. Such acetone quantities are not attained by bubbling for 2 minutes.)

From 1000 to about 100 μg: Finely crystallized, smoothly dispersed, yellow precipitate in the DNPH solution. The highest values cause almost immediate precipitation, at lower values it occurs within one minute. If before bubbling the reagent was limpid, shaking with carbon tetrachloride is unnecessary; however, if shaken, the carbon tetrachloride layer becomes a bright yellow color.

From 100 to about 50 μg: No precipitate. If compared with a control DNPH solution of identical thickness of layer, the hydrazoneous solution will be found to be of a darker hue. After shaking with carbon tetrachloride the color intensity of this layer is more pronounced as compared to the aqueous phase. An exact comparison is impossible because the two colors are not indentical; the carbon tetrachloride layer is more greenish.

From 50 to about 10 μg: No precipitate in the reagent. After shaking with carbon tetrachloride this layer assumes a greenish-yellow color, and its intensity, as compared to the DNPH phase, decreases proportionally to the quantity of acetone.

8 μg: May give rise to some doubt if not shaken with sodium hydroxide; otherwise there will remain a slight yellow coloring of the bottom layer as opposed to the blank; the latter is therefore not necessary except if, as already mentioned, the test be performed in electric light.

The above serves only as an outline; it may vary somewhat according to testing procedures. The most important distinction is: greater color intensity in the carbon tetrachloride layer than in the aqueous phase (before the addition of hydroxide) indicates an abundant content of acetone, *i. e.* more than 50 μg, whereas identical or less intensive coloring indicates quantities below 50 μg. Amounts of acetone yielding yellow precipitates occur rather infrequently in patients.

SOURCES OF ERROR

Since only non-ionised compounds penetrate the alveolar wall and appear in the expired air the number of pit-falls is rather restricted. The following substances have been examined: *Ethanol* in very high concentrations (which are not encountered in patients) leave a pale yellow coloring of the carbon tetrachloride. The coloring disappears after shaking with sodium hydroxide. *Metha-*

gives no color reaction. *Acetaldehyde* s exactly like acetone; equimolar solu- of the two compounds yield identical ring of the carbon tetrachloride layer, mixtures of the two compounds yield lor intensity according to the total of molar concentration. Larger concen- ons of acetaldehyde — in contradistinc- to acetone — yield a positive Fehling's after absorption in water. Traceable ldehyde concentrations in human ex- l air may be found after intake of al- l, particularly if the patient previously been subjected to treatment with tetra- ltiuramidsulphide (Antabuse) (Hald & bsen, 1948; Asmussen, Hald & Larsen, 8). In one of our patients who was ed with Antabuse a positive reaction manifest for two days after the intake lcohol.

A COMPARISON BETWEEN OTHER ETHODS AND AUTHORS' METHOD

able I recording earlier methods shows t variability in the sensitivity of the ods. Based on our own controls of sen- ities we selected such reactions as were n apparent sensitivity which was at least lar to that of our own method. These tions were further examined and com- d with the DNPH method by making oneous air bubble directly through the erent reagents or through water which then tested for acetone by means of the ents. The time of bubbling required l the reactions became faintly positive determined. The actual sensitivity of a n method is conversely proportional to period thus measured.

As expected it is an advantage if the expired air bubbles through a reagent which reacts with acetone, and this is preferable to performing the reaction directly on distilled water which absorbs the acetone. The latter method requires more time before equal quantities of acetone are retained, the "binding capacity" is inferior and, immediate performance of the test is required or the acetone will evaporate. Within reasonable periods Lieben's iodoform method is, as mentioned, almost as sensitive (10 μg per 2 ml) as the DNPH method, but the acetone must be absorbed in water. *Frommer's salicylaldehyde method* is excellent and traces the same quantities of acetone (8 μg) as DNPH, but requires initial absorption of the acetone in water. Sensitivities of these two methods under the given conditions were accordingly found to be much inferior to that of the DNPH method. *Scott-Wilson's mercuric cyanide reagent* is toxic — although the formation of complexes probably reduces the toxicity considerably. But one would hesitate to let a patient breathe directly through a tube if aspiration of the reagent is not thoroughly safeguarded. The sensitivity is much inferior to that of the DNPH reaction if the acetone is first absorbed in water. However, the Scott-Wilson reaction is found to be less sensitive than the DNPH even after bubbling directly through the reagents. As the sensitivity of the Scott-Wilson reagent to the acetone actually absorbed in the aqueous phase is higher (2—3 μg in 2 ml) than that of the DNPH reagent, the latter must react with the acetone more rapidly and thus more quantitatively than the mercuric cyanide reagent.

CLINICAL APPLICABILITY OF THE METHOD

The DNPH method is built up on the basis of practical experience from hospitalized acidotic diabetics. Concurrently with the DNPH test on expired air, fresh specimens of urine were examined for ketones by Gerhardt's method (*i. e.* dropwise addition of ferric chloride solution; a purplish coloring indicates acetoacetic acid) and by Legal's method (*i. e.* additions of small quantities of pulverized sodium nitroprusside, one fifth volume of 10 per cent potassium hydroxide solution, acidification with 30 per cent of acetic acid solution; a permanganate coloring is retained when acetone and/or acetoacetic acid are present).

The results from the three tests were found to harmonize in all cases but two. In the two deviating cases the DNPH method gave a positive result whereas Legal's reaction was negative; Gerhardt's reaction was faintly positive in one, negative in the other case.

When the method had been elaborated it was tested on diabetic out-patients[1] as follows:

Patients producing 24-hour-urine specimens which reacted positively to Legal's and/or Gerhardt's tests were subjected to the DNPH test on their expired air. Subsequently the patients were asked to empty their bladders; after 30—60 minutes a fresh urine specimen was obtained and directly tested for ketones after Legal's and Gerhardt's tests; the sugar was determined after Benedict's method.

[1] We are indebted to Chief Physician P. Schultzer, M. D., the Out-Patients' Department, The Municipal Hospital, Copenhagen.

These examinations were carried out by a technician to whom the DNPH method had been briefly explained. To give an impression of the method's applicability in the routine the authors were excluded from performing any of the tests.

The first three examinations were rather deceptive, but after the initial difficulties had been overcome the findings were as seen from Table II.

DISCUSSION AND CONCLUSION

It will be observed from Table II that there was general agreement between examinations of expired air and examinations of urine. Positive reactions were by far more frequent after Legal's method than after Gerhardt's, which is in accordance with the findings of Lestradet (1951) *viz.* that Legal's test — besides being capable of tracing acetone — also is more sensitive toward acetoacetic acid than Gerhardt's test. In one patient (No. 19) both urine reactions were negative, but the DNPH method gave a positive, although faint, result. In the two previously mentioned hospitalized patients and in one out-patient the results of the DNPH method thus proved more satisfactory than the urine reactions. Similar cases would no doubt have been more numerous if the out-patients were not chosen on the basis of the content of acetone and acetoacetic acid in their 24-hour-urine specimens. No examinations were made on expired air from patients whose 24-hour-urine showed no acetone or acetoacetic acid, and "borderline" cases were therefore rarely met with.

ble II. *Findings from 23 consecutive s on expired air and fresh urine speci- s from 22 diabetic out-patients whose our-urine from the preceding day re- ed ketones. (3 and 7 refer to the same ent tested on different days.) The mate- includes 16 women and 6 men aged 12—72 years.*

			Fresh urine specimens			Expired air (DNPH method)	
Insulin treatment	Resting blood sugar mg in 100 ml	Sugar determination Benedict's method	Determination of ketones. Gerhardt's	Determination of ketones. Legal's	After shaking with carbon tetrachloride	After renewed shaking with 5 N sodium hydroxide
+	243	+	0	+	++	++
+	253	+	0	+	++	+
0	337	++	+	+	+++	+++
+	268	++	+	+	+	++
+	298	++	0	+	+	+
0	304	++	0	+	++	+
+	272	++	+	+	++	+
0	296	++	0	+	++	++
0	115	+	+	++	++	++
+	243	+	+	+	+	+
+	>385	++	+	++	+	+
+	258	+	0	+	+	+
+	262	+	+	+	+	+
+	341	++	0	+	++	++
+	288	+	+	+	++	++
+	338	+	+	+	+++	+++
0	242	+	+	+	++	++
0	206	+	+	+	0	+
0	216	+	0	0	0	+
0	230	++	+	+	+	+
+	290	++	0	+	+	+
+	210	++	+	+	+	+
0	264	+	0	+	+	+

Having used the DNPH method for some e we find it justifiable to sum up ad- tages and drawbacks as follows: the re- nts are easily prepared and preserved, are non-toxic and inexpensive. Instruments are simple and available in any laboratory. The test is sensitive, easily readable and applicable, *e. g.* neither filtration nor heating is required. The method has only few and insignificant sources of error. Storing has only a slight influence on the coloring. As already mentioned in the introduction the DNPH method offers practical as well as theoretical advantages over the determination of ketones in urine. But it has also definite advantages over the demonstration of acetone and acetoacetic acid in the blood as the process of blood sampling for obtaining serum for this purpose is time-consuming and laborious. Presumably the test must be applicable in hospitals and also prove useful to the general practitioner and the diabetic himself.

It should finally be emphasized that the acetone excretion indeed is no exact expression of the patient's ketonemia; an analysis for acetoacetic acid in the urine can therefore scarcely be omitted in all cases; some cases require more exact examinations of shifts in the electrolyte balance. But provided the blood sugar as well as the exhaled acetone be closely watched in comatous diabetics the routine should probably be to wait until the patient urinates before any examination of the urine is performed.

In order to further elucidate these conditions investigations have been instituted to establish the quantitative relation between the expired acetone and the total quantity of ketones in the blood.

SUMMARY

A method is described by which acetone in expired air is determined: for 1—2 minutes expired air is bubbled through 2 ml of a solution of 0.2 per cent 2,4-dinitrophenyl-

hydrazine in 2 N hydrochloric acid. The acetone-dinitrophenylhydrazone thus developed is extracted by 2 ml of carbon tetrachloride. Yellow coloring of the latter indicates acetone. It is recommended that 2 ml of 5 N sodium hydroxide be added when determining small quantities of acetone thus ensuring a colorless blank, — which may usually be omitted.

The method is sensitive to 0.008—0.010 mg of acetone. Expired air from normal subjects gives no positive reaction. The only compound in expired air which may be confused with acetone is acetaldehyde which is found only in patients after intake of alcohol, particularly consequent on treatment with Antabuse. This is illustrated by a single case.

In 24 diabetics with a presumable ketonemia a comparison was made of the ketone content in the urine and results from the method here described. 21 cases conformed to pattern and yielded positive results; in three cases Gerhardt's and Legal's urine reactions were found to give negative results, whereas the test for acetone in expired air was positive. This procedure is thus found more sensitive than the methods hitherto applied for the determination of ketones in urine, and offers various practical advantages, particularly in cases of comatous diabetics.

ACKNOWLEDGMENT

We thank Mrs. Inge Christiansen, Mrs. Lis Holmelund, Miss Aase Johansen and Mr. John Graae, M. A., for valuable assistance.

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CALCULATION OF THE BODY SURFACE AREA BY PHOTOGRAMMETRY

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(Received for publication June 15, 1954)

The first paper on measurement of the body surface area was published in 1793 by John Abernethy (cited by Du Bois). He calculated the surface area of the trunk according to length and average circumference, and the surface area of the head and the limbs by covering the head with paper, which he then removed, flattened out and measured. He measured a man, 5 feet 6 inches high, and found a figure of 2700 square inches.

In 1879 Meeh carried out direct measurements by approximately the same method. He measured sixteen individuals — six adults and ten children. Based on these measurements Meeh set up the following formula for the surface area, expressed as a function of the weight: —

$$S = 12.312 \sqrt[8]{W^2}$$

In subsequent years a number of other investigators endeavoured partly to make their own formulas, partly to correct the one set up by Meeh (Bouchard 1887, Hecker 1894, Miwa & Stöltzner 1898, Sichiff 1902, Lissauer 1902, Brocha 1907; cited by Du Bois and Medicinske Tall).

The formula of Meeh was maintained, however, and was used on the whole for about thirty-five years. Rubner, for example, used Meeh's formula in his studies on metabolism.

In 1918 D. Du Bois performed new direct measurements of five adult individuals. He covered the test individuals with paper, cut this into small strips, which he then laid flat on a large photographic plate, which he had weighed beforehand. The plate was exposed, and the unexposed parts were cut out and weighed.

The following year E. F. Du Bois measured five more adults by the same method. On the basis of these ten measurements a so-called linear formula was set up, based on nineteen different measurements of circumference and length of various parts of the head, the trunk and the limbs. In the formula were incorporated seven different factors of correction.

According to this formula the surface area was calculated in a number of individuals, and Du Bois & Du BBois arrived at the following formula on the basis of these measurements, in which the surface area was expressed as a function of weight and height: —

$$S = 71.84 \times W^{0.425} \times H^{0.725}$$

Thus, this formula is actually based solely on ten direct measurements of adult individuals.

Hannon (cited by Du Bois & Duncan) has prepared a monogram for children according to this formula.

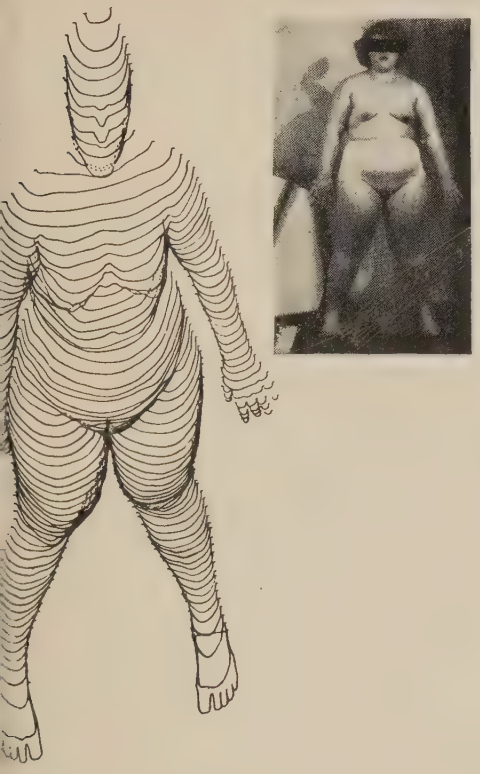

Fig. 1.

ubsequently several other investigators
e made calculations of the surface area,
ly according to the linear formula, partly
lirect measurements on almost the same
ciple as that applied by Du Bois (Bene-
1916, Pfaudler 1916, Wörner 1923,
ahira 1925 (cited by Du Bois). The
es found for the surface area deviate
art considerably from the values found
he Du Bois formula.
he formula of Du Bois, however, is the
used almost everywhere even today.
the present paper a new method of
ct surface area measurement is sub-
ed.

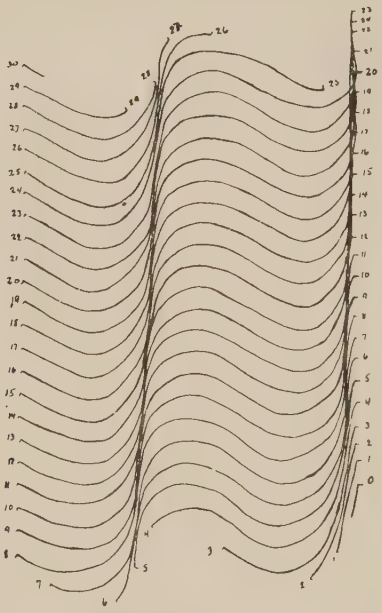

Fig. 2.

The method is based on terrestrial photogrammetry, and has been prepared in collaboration with "Norges Geografiske Oppmåling" (Geographical Survey of Norway), their equipment having been used for the measurements.

Stereograms have been prepared of the entire body of the test individuals from behind and from the front. According to the stereoscopic model, curves have been traced in an autograph, with incisions in the horizontal plane at small equidistances (Fig. 1). All constructions have been made by Petter Vold (topographer).

All lengths of the curves have been measured with a curve measure, and the total curve length has been multiplied by the equidistance. In addition comes the area of the

soles of the feet and the crown of the head, which have been measured with a planimeter. The error of multiplying the curve length directly by the equidistance instead of by the actual curve distance is diminished the shorter the equidistance.

In order to check the accuracy of the method a plate with a known area is used. The plate has been bent and twisted in various planes, and thereupon photographed, and curves have been prepared with the same equidistance as used in the test individuals (Fig. 2).

An area has been found by the measurement which is 1.3 per cent lower than the correct measurement. The average curve distance has been calculated on the basis of the displacement of the curves, and by using this instead of the distance the calculation shows a deviation of merely 0.17 per cent. Up to the present the method described above has been used for calculation of the surface area in eight individuals only (3 normal boys, 4 men with greatly varying body configuration, and 1 extremely overweight women).

The result of the calculations has been compared with the surface area calculated by the formulas of Meeh and Du Bois. By the Meeh formula values have been obtained of up to 35 per cent higher than those found by the method submitted in the present paper, while the values according to the Du Bois formula deviate from —8 per cent to +2.5 per cent.

SUMMARY

In the present paper the author submits a new topographical method for measuring the surface area of the body.

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N THE DETERMINATION OF CREATININE IN PLASMA BY THE JAFFÉ REACTION, AFTER ADSORPTION TO LLOYD'S REAGENT[1]

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Many attempts have been made to develop reliable method for the determination of atinine in biological fluids. There seems be good agreement that the Jaffé's alkaline rate color reaction is the most convenient this purpose. This reaction, however, is completely specific, as it is also given a few other chromogens, generally re-red to as "pseudocreatinines". Hunter & mpbell (1917) claim that glucose is re-nsible for nearly all of the color caused pseudocreatinine, whereas Lauson (1951) ds glucose less important than other romogens.

Since Folin & Wu (1919) published their ginal method for precipitation of blood teins, several authors, (Wu 1922, Brod Sirota 1948) have tried to improve this thod to obtain the lowest possible amount pseudocreatinine in the filtrate. Haugen 953) has compared some of the modifi-ions and concludes that the method of od & Sirota (1948) is to be preferred, this method gives 100 per cent recovery added creatinine, and very low pseudo-atinine values.

Hare & Hare (1949) state that tungstic, picric, and trichloracetic acid, as well as cadmium, zinc or iron filtrates of serum or plasma, continue to develop color with alkaline picrate for two hours or more, *i. e.* they all contain pseudocreatinine.

In 1935, Borsook introduced Lloyd's reagent (Fuller earth) as an improvement in the determination of creatinine. From an acid serum or plasma filtrate, Lloyd's reagent adsorbs all the creatinine, but none of the pseudocreatinines, or at least only an ignorable amount. When transferred to an alkaline medium, the creatinine is eluded from the reagent. This method is further developed by Hare (1950) and later modified by Blegen & Haugen (1953).

Lauson (1951) has compared Hare's method with several other methods that do not make use of Lloyd's reagent. He concludes that the adsorption-elution method is superior to the others, and that it gives 100 per cent recovery of added creatinine.

The present investigation is a critical examination of the various steps in the method described below (a modification of Borsook's method), with special regards to the use of Lloyd's reagent and the details in connection with absorption to and elution from this reagent. A comment is given to the precipitation of the proteins.

[1] Aided by a grant from A/S Freia Chokolade-riks Medicinske fond.

METHOD

The method is a modification of Borsook's method (1935).

Reagents:

1 N trichloracetic acid.

Lloyd's reagent (Fuller earth) (B. D. H. or Hartman-Leddon Co.).

Alkaline-picrate reagent, consisting of 5 parts of saturated, neutralized picric acid, 12 parts of H_2O, and 1.0 part of 5.5 per cent NaOH. (The saturated picric acid is neutralized in the following way: To the chosen amount of saturated picric acid solution is added a strong solution of NaOH (20 per cent or more), drop by drop until alkaline reaction (indicator paper). Then more picric acid solution is added until neutral reaction.)

Procedure for serum or plasma:

1. Precipitation of proteins:

To 5 ml of serum or plasma (moderate hemolysis does not play any significant rôle) are added 5 ml of H_2O, and mixed well.

10 ml of trichloracetic acid are added, and the tubes are shaken well. After 10 minutes the tubes are shaken again, and the content filtred under watch glass cover.

2. Adsorption to Lloyd's reagent:

10 ml of filtrate + 200 mg of Lloyd's reagent (a spoon containing approximately 200 mg was used) are shaken in glass-stoppered test tubes. After 10 minutes, during which time they are gently shaken every minute, the tubes are centrifuged for 5 minutes at about 800 · g, and the supernatant is carefully decanted and discarded.

3. Elution and color reaction:

After decanting, 10 ml of alkaline picrate reagent are added to the test tubes. The tubes are shaken again, and then gently shaken now and then for 10 minutes, and centrifuged for 5 minutes, at about 800 · g.

The supernatant is read against a blank in a spectrophotometer (Beckman, model B or model DU, or Uvispec) at 490 mμ.

The blank is prepared as described above, substituting water for serum or plasma.

4. Calculation:

The concentration of creatinine in serum is derived from a standard curve prepared from known concentrations of creatinine in aqueous solution, treated the same way as serum.

Procedure for urine

The urine is diluted to contain 1—4 mg creatinine per 100 ml, (generally 1 : 100) and treated as described for serum.

It is permissible to omit the Lloyd's reagent when dealing with urine, but the values then have to be derived from a standard curve prepared without Lloyd's reagent.

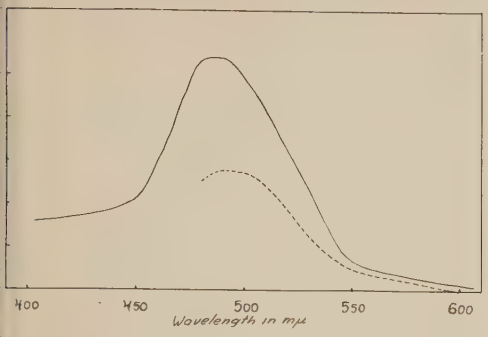
EXPERIMENTAL

The method has been examined for the following moments, using pure solutions of creatinine when not otherwise stated.

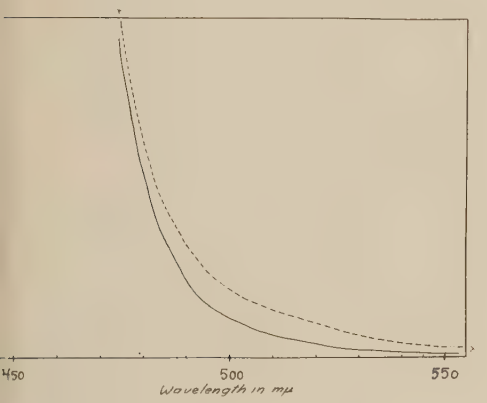
1. Color absorption curve and choice of wave length

Previous investigators without exception read the creatinine-picrate color at 500—520 mμ. Creatinine solutions 1—4 mg per cent, treated with alkaline picrate and read against a picrate blank, however, show a definite absorption maximum at a plateau between 485 and 490 mμ (Fig. 1), (1—4 mg per cent in the sample give a concentration of 0.25 to 1.0 mg per cent of creatinine in the final solution, when the procedure is carried out as described above). At 500 mμ, the decline of the absorption curve is already so steep that small alterations in the wave length will influence the results.

Absorption curves of creatinine-picrate solutions, read against a water blank, showed an increase in optical density with decrease in wave length, sharply rising below 500 mμ, and so does the alkaline picrate blank, when read against a water blank (Fig. 2). Read-



g. 1. Extinction curve of 0.5 mg per cent crea-ine in alkaline picrate solution 0.04 n, read against an alkaline picrate blank.
—— Beckman "B" & "DU" and Uvispec (10 mm cuvettes).
- - - Coleman jr. (8 mm cuvettes).



. 2. Extinction curves for an alkaline picrate nk (alkalinity 0.04 n) and the same solution with mg per cent creatinine, both read against a water blank.
—— Picrate blank.
- - - Creatinine solution.

s in a Coleman Jr. spectrophotometer, in Beckman B as well as DU model, and in Uvispec, all gave the same results.

A change in the NaOH concentration of alkaline picrate solution will influence

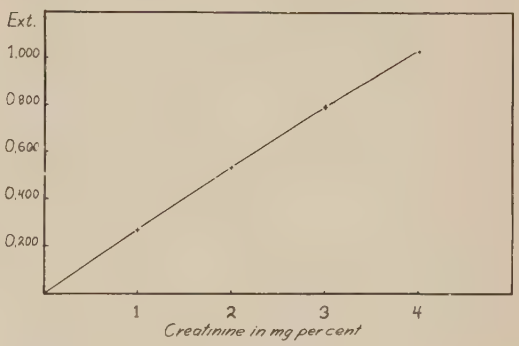


Fig. 3. Standard curve for creatinine solutions from 1 to 4 mg per cent, treated according to the method described in this paper.

the extinction values (see § 5), but will not alter the absorption maximum.

It was decided to read the color at 490 mμ, and always against an alkaline picrate blank.

2. De Beer's law

The creatinine-picrate color approximately obeys de Beer's law, up to 1.0 mg per cent of creatinine in the final solution, corresponding to a concentration of 4 mg per cent in the sample. If higher creatinine concentrations are to be examined, the solution should be diluted. The extinction curve for concentrations up to 4 mg per cent in the sample is given in Fig. 3.

3. Temperature

As stated by previous authors, temperature influences the Jaffé reaction. It is shown that the influence varies considerably with the alkalinity of the final solution. With a normality of 0.04 n NaOH in the final solution, the difference in optical density from +18° C to +29° C ranges to about 2 per cent. If the alkalinity is increased to 0.10 n NaOH, the difference in optical

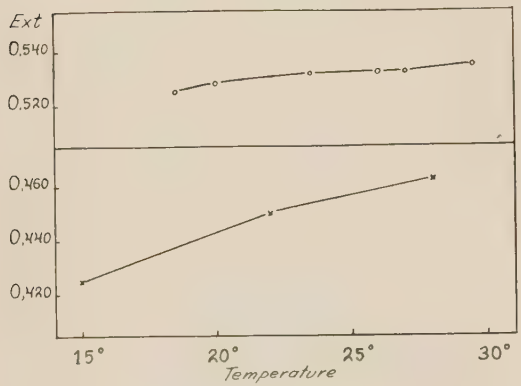


Fig. 4. Influence of temperature at different alkalinities in the final solution.
o——o 0.5 mg per cent creatinine in alkaline picrate solution 0.04 n.
x——x 0.5 mg per cent creatinine in alkaline picrate solution 0.11 n.

density over the same range of temperature increases to about 8 per cent (Fig. 4).

When using an alkalinity of 0.04 n NaOH, the variations at room temperature (21°± 3°) are negligible.

4. *Time factor for the color development*

Pure solutions of creatinine, treated with alkaline-picrate, gave a color fully developed within 12 minutes and stable for at least 90 minutes. Plasma or serum filtrates gave the same stability for 90 minutes. Observations after 15 hours showed varying and highly irregular drops in extinction values.

No precautions were taken against the influence of light. No special experiment has been performed on this point, but the different experiments have been made in daylight as well in artificial light, with no change in the extinction values from time to time.

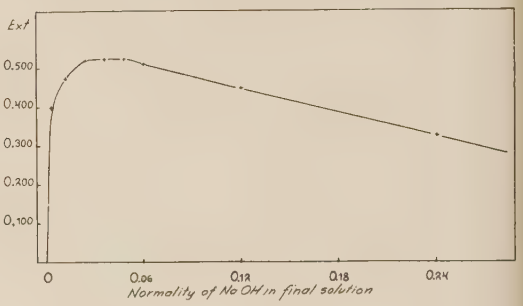


Fig. 5. Extinction curve of 0.5 mg per cent creatinine in alkaline picrate solution with varying excess of NaOH in final solution.

5. *Alkalinity of the picrate solution*

When the normality of NaOH in the alkaline-picrate solution was changed, the optical density was greatly influenced.

In order to study the variation of the NaOH concentration and its influence on the optical density, the following experiment was performed:

Alkaline picrate reagents were made with NaOH solutions up to 20 per cent. This corresponds to an excess of NaOH in the final solution ranging from 0.0 to 0.24 n., which was controlled by titration. To 10 ml of alkaline picrate reagent was added 0.5 ml of a 10.5 mg per cent of creatinine solution, giving a final concentration of 0.5 mg per cent of creatinine, which corresponds to 2 mg per cent in the sample, if the procedure had been carried out as described under method.

The results are given in Fig. 5. There is a marked increase in optical density up to 0.03 n NaOH and a small plateau from 0.03 to 0.05 n. From 0.05 n to 0.24 n, there is an even decrease in extinction values.

This is in good agreement with the findings of Bonsness & Tausky (1945).

Previous investigators have all made up alkaline picrate reagent with unneutralized picric acid. As the content of picric d in a saturated solution will vary greatly th the temperature, the excess of OH^- is in the final solution of alkaline picrate l also be subject to variations when the reent is made with unneutralized picric acid. at the excess of OH^- ions is conditional the optical density, and not the total ount of NaOH, was shown by the following experiment:

Alkaline picrate reagents were made up cording to the method described in this per, but with different solutions of aOH, varying from 2 to 10 per cent. In e experiment all reagents were made with utralized, and in an other experiment with neutralized picric acid. Then creatinine s added in an amount of 0.5 mg per 100 ml agent, and the samples read against their spective alkaline picrate blanks. When the al solutions were titrated with 0.10 n HCl, was found that the same excess of NaOH ways corresponded to the same extinction lues (Fig. 6). In all these experiments, e Lloyd's reagent was omitted.

It was decided always to make the alkae picrate solution with neutralized picric id. It was further decided to make the alline picrate reagent with 5.5 per cent solun of NaOH. This percentage does not rrespond to the plateau of the curve, but e reason for this choice will be further xplained in § 7.

Protein precipitating reagents

The trichloracetic acid filtrates were comared to the Wu filtrates (Wu 1922), proced according to the following procedure: 5 ml of plasma or pure creatinine solutions ranging from 1 to 4 mg per cent, and 5 ml of H_2O were mixed. Then 5 ml of 2/3 n H_2SO_4 and 5 ml of 5 per cent sodium tungstate solution were added. The tubes were shaken and allowed to stand for 10 minutes, whereafter they were shaken again, and filtred under watch glass cover. To 10 ml of filtrate was added 1 ml of saturated oxalic acid and 200 mg of Lloyd's reagent, and the tubes were further treated as described under Method.

When read in the spectrophotometer, samples made from Wu filtrates showed no significant difference from those made from trichloracetic acid filtrates.

On the other hand, trichloracetic acid filtrates have many advantages as compared with the Wu filtrates.

a) After precipitation with trichloracetic acid, the filtration rate is far higher than after precipitation according to Wu.

b) The filtration yield is much greater with the trichloracetic acid method, than with the Wu method. (With the Wu method it is hard to obtain 10 ml of filtrate from the 20 ml of precipitated solution, whereas the trichloracetic acid filtrate far exceeds this quantity.)

c) The trichloracetic acid gives an absolute protein-free filtrate, whereas the Wu filtrate must be handled very carefully to give a satisfactory result. In plasma filtrates produced according to the Wu method, small amounts of unprecipitated protein often caused great errors in the determinations. The Lloyd's reagent got a fluffy appearance, and the extinction values obtained were highly unreliable.

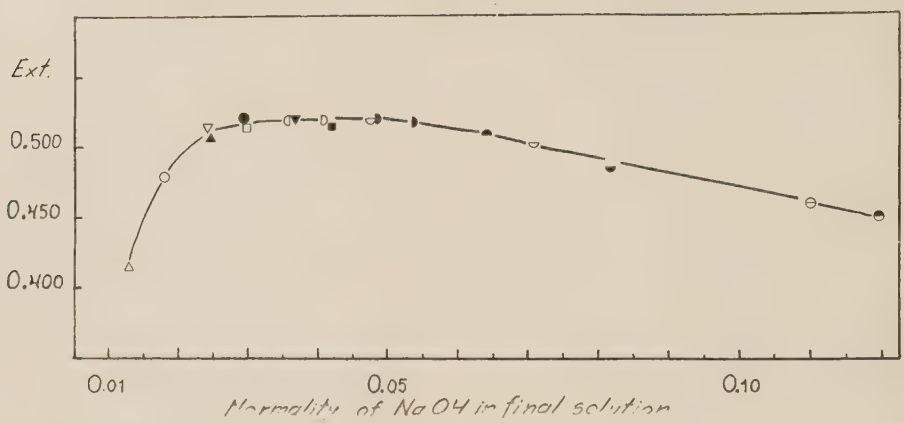


Fig. 6. Extinction values for 2 mg per cent creatinine solutions, treated according to the method described but with varying percentage of NaOH solution added to the picrate reagent.

Open symbols: Prepared with unneutralized picric acid.
Cloced symbols: Prepared with neutralized picric acid.

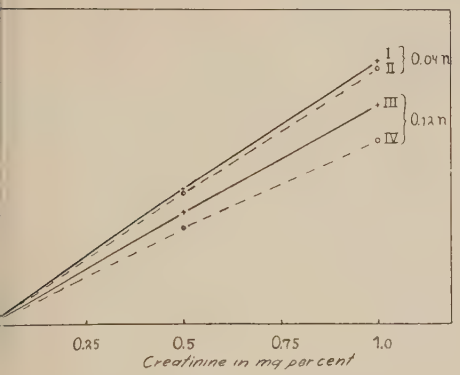
▲ △ } 2 per cent NaOH
● ○ } 2.5 per cent NaOH
▼ ▽ } 3 " "
■ □ } 3.5 " "
◖ ○ 4 " "
◗ ○ 4.5 " "
◓ ◯ } 5 per cent NaOH
◒ ◡ } 7 per cent NaOH.
◉ ⊖ } 10 " "

Experiments were also carried out using aqueous creatinine solutions, and with water substituting the sodium tungstate solution. This had no significant influence on the results, so the tungstate seems not to influence the creatinine-picrate color.

7. Lloyd's reagent.

Previous investigators (Lauson 1951, *et al.*) claim that Lloyd's reagent adsorbs all the creatinine from acid solutions, and that all the creatinine is eluded from the reagent in alkaline solutions. In order to elucidate whether the creatinine-picrate color is adsorbed to Lloyd's reagent in alkaline milieu, a comparison was made between the color of alkaline creatinine-picrate solutions without and with Lloyd's reagent added. A moderate but significant difference was found, indicating a color adsorption to the reagent. The difference is directly proportional to the amount of Lloyd's reagent, and related to the alkalinity of the solution. With increasing alkalinity, there was an increasing amount of color adsorbed.

With an alkalinity of 0.04 n in the final solution, the apparent loss of creatinine only amounted to about 2 per cent of the total when 200 mg of Lloyd's reagent were added,



7. Extinction curves for creatinine solutions 0.25 to 1.0 mg per cent in alkaline picrate solution.

Alkalinity 0.04 n, not treated with Lloyds reagent.

Alkalinity 0.04 n, treated with 200 mg of Lloyds reagent.

Alkalinity 0.12 n, not treated with Lloyds reagent.

Alkalinity 0.12 n, treated with 200 mg of Lloyds reagent.

reas with an alkalinity of 0.11 n NaOH, loss was increased to between 8 and 9 cent. Further, the creatinine-picrate color orbed to Lloyd's reagent in alkaline solu- is proportional to the total amount of tinine in the solution.

ig. 7 shows the drop in extinction values n creatinine picrate solutions of dif- nt alkalinities are treated with increasing unts of Lloyd's reagent. The experiment performed in the following way: To line picrate solutions of 0.042 n alka- y, was added creatinine in amounts rang- from 0.25 to 1.0 mg per 100 ml solution. extinction values are given in curve I. en the same samples were shaken with mg of Lloyd's reagent per 10 ml of solution and centrifuged, the extinction values in the supernatant dropped to those indicated in curve II.

Curve III represents the values obtained when the alkalinity of the picrate solution was increased to 0.12 n, and curve IV when these samples where treated with Lloyd's reagent in the same way. All samples were read against an alkaline picrate blank of the same alkalinity as the sample. The blanks used for curves II and IV were also treated with Lloyd's reagent. There was, however, no significant difference in optical density between the blanks treated with Lloyd's reagent and those not treated with this reagent. This indicates that Lloyd's reagent does not adsorb sodium picrate in alkaline solutions.

The experiment makes it clear that Lloyd's reagent adsorbs some creatinine-picrate color in alkaline solutions, and that the amount of creatinine-picrate color adsorbed is proportional to the total amount of creatinine in the solution. The adsorption is also related to the alkalinity of the final solution, and seems to be almost negligible when the alkalinity is about 0.04 n.

This alkalinity also falls within the range where the creatinine-picrate color gives maximum extinction values (Fig. 5).

That the amount of creatinine- picrate color adsorbed to Lloyd's reagent is directly proportional to the amount of Lloyd's reagent, is shown in Fig. 8. Solutions of 0.50 mg per cent creatinine in alkaline picrate reagents with a final alkalinity of 0.04 and 0.11 respectively, were treated with increasing amounts of Lloyd's reagent from 100 to 400 mg per 10 ml solution. The extinction

values obtained in the 0.04 n solution are given in the upper curve, and those in 0.11 n solution in the lower curve. The difference in the loss of creatinine-picrate color in different alkalinities is clearly demonstrated in this experiment too.

As stated in a previous section (§ 5), the Jaffé reaction is dependent upon the alkalinity of the final solution. When the trichloracetic acid filtrate is treated with Lloyd's reagent to adsorb creatinine, there will always be a small amount of acid equivalents transferred to the final alkaline solution by the Lloyd's reagent. This amount of acid was titrated and found to vary between 0.29 and 0.33 mE, depending upon how carefully the supernatant is decanted. (Titration values from 200 mg Lloyd's reagent.) When Lloyd's reagent was increased to 400 mg, the acid equivalents also increased to about the double. As serum *eo ipso* neutralizes a small part of the trichloracetic acid, the acid rest transferred from the serum filtrates will be a trifle smaller than the rest transferred from the blanks.

This amount of acid components will decrease the excess of hydroxyl ions in the alkaline picrate reagent. From Fig. 5 it is evident that if the alkaline picrate reagent is made up with for instance 10 per cent NaOH, corresponding to an alkalinity of 0.12 n in the final solution, the amount of acid transferred with Lloyd's reagent will lower the alkalinity to about 0.09 n (from 1.2 mE to 0.9 mE per 10 ml solution) and consequently increase the extinction values by about 8 per cent.

This might be an explanation of the fact that previous investigators, who made their alkaline picrate reagent with 10 per cent

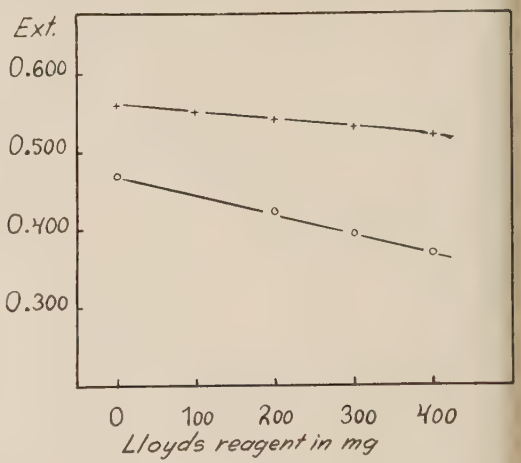


Fig. 8. Influence of Lloyds reagent in varying amounts on the extinction values of 0.5 mg per cent creatinine in alkaline picrate solution.
+ - - - + Alkalinity 0.04 n.
o - - - - - o Alkalinity 0.11 n.
Ordinate: Extinction values.
Abcissa: mg of Lloyds reagent.

solution of NaOH, claim that the creatinine is completely eluded from Lloyd's reagent in alkaline solutions. As mentioned above, there is an apparent loss of about 9 per cent of the creatinine, due to the adsorption to 200 mg of Lloyd's reagent in an alkaline solution of about 0.12 n. When the extinction values, however, are brought to increase about 8 per cent by the acid transferred to the final solution with the same amount of Lloyd's reagent, there will be a fictitious recovery of almost 100 per cent.

As long as the alkalinity of the picrate solution is kept on the decline of the curve, variations in the amount of acid transferred with Lloyd's reagent will influence the extinction values to some degree. When the excess of NaOH is adjusted in a way that brings the final concentration to fall on the

au of the curve, *i. e.* between 0.025 and
) n, this source of error will be avoided.
was therefore decided to make up the
ine picrate reagent with 5.5 per cent of
)H solution which, when the transferred
is considered, theoretically will bring
final excess of NaOH to about 0.04 n.
was confirmed by 100 replicates that
titrated with 0.1 n HCl.
wo different brands of Lloyd's reagent
tested, one from B. D. H., and one
n Hartman-Leddon Co., U. S. A. There
no significant difference between these
ds.

Determinations of creatinine in plasma and recovery of added creatinine

lasmas from 9 normal persons have been
nined, and the creatinine values have
derived from standard curves made
pure solutions of creatinine. The values
ined are given in Table I. Standard de-
on of the method was calculated in per
of mean. From 87 single determinations
standard deviation was calculated to
.1 per cent, according to the formula

$$\sigma = \frac{\sqrt{\Sigma\, nd^2}}{N \div 1}$$

o 6 plasmas was added 1 mg per cent
tinine, and the recovery was determined.
values are given in Table II. The mean
e of the recovery was calculated to 98.7
cent, with a range of 96 to 101 per cent.
standard deviation was calculated to
.4 per cent (130 single determinations).
heparin plasmas were left in the re-
erator for 24 hours and the creatinine
es determined again. In 3 plasmas the

Table I. *Creatinine values in serum from healthy subjects.*

Subject	No. of determinations	Mg per cent of creatinine	
		Mean	Range
NN	12	0.92	0.90—0.92
NN	12	0.72	0.71—0.73
ER	2	1.21	1.21—1.21
AA	2	0.72	0.71—0.72
Stud.	10	0.88	0.86—0.89
Stud.	15	0.95	0.94—0.97
BB	12	1.05	1.04—1.06
CC	10	0.92	0.91—0.92
EH	12	0.92	0.91—0.93

Table II. *Recovery of added creatinine to plasma.*

Subject	Creatinine mg per cent	Added Creat. mg	Creatinine recovered		No of determinations
			Mean	Range	
NN	0.92	1.00	0.99	0.97-1.00	15
NN	0.72	»	0.98	0.97-1.00	15
Stud.	0.88	»	0.99	0.95-1.00	25
Stud.	0.95	»	0.97	0.96-0.98	25
BB	1.05	»	0.99	0.97-1.01	18
CC	0.92	»	0.98	0.97-1.00	12
EH	0.92	»	0.99	0.98-1.01	20

values were decreased by 3—5 per cent, and in one plasma the values were increased by about 3 per cent.

SUMMARY

A method for determination of creatinine in blood and urine is described. The method is based on previous works by Borsook (1935) and Brod & Sirota (1948) and includes adsorption and elution on Lloyd's reagent, with a final color reaction by the Jaffé method. The different steps of the method are critically examined under various conditions, and the results discussed.

1. The most convenient wave length for reading the creatinine picrate color in the spectrophotometer is found to be 490 mμ.

2. The sensitivity of the color development to variations in the temperature is diminished by adjusting the final alkalinity to 0.03 to 0.045 n.

3. The maximum color development is found to take place within the same range of alkalinity (0.03—0.05 n).

4. The creatinine-picrate color is found to be adsorbed to Lloyd's reagent to a minor extent depending on the alkalinity of the milieu. With increasing alkalinity there was an increasing adsorption. A minimum adsorption was found at an alkalinity of 0.03 to 0.04 n.

5. The standard deviation of the method is found to be ± 1 per cent, and the recovery of added creatinine to plasma (1 mg per cent) is found to be 98.7 per cent, with a standard deviation of ± 1.4 per cent.

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RAPID CLINICAL METHOD FOR THE DETERMINATION CALCIUM IN SERUM AND OTHER BIOLOGICAL FLUIDS

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or the determination of hardness in er, that is for the determination of cal- n and magnesium, complexones, ethylene- ine-tetraacetic acid (EDTA), have been in industrial laboratories for many s. Together with the metal ions EDTA s, especially in alkaline pH, a stable atcomplex (intrinsic complex anion) warzenbach & Biedermann 1948). Erio- meschwarz T is used as specific in- tor (Biedermann & Schwarzenbach 8). In suitable pH — 9.5—10 — this cator is blue, as free EDTA is present e solution. The color turns slightly red- t, especially because of the influence of free magnesium ions. The free calcium give a more indistinct color shift. If w dye is added to the indicator, for in- ce methyl red as background color, the r changes from green to red, which is easy to notice.

he clinical chemist have also observed many advantages of the complexones, many methods for the determination of ium in blood have already been presented rding to this principle. (Nielsen 1952, t 1952, Sobel & Hanok 1951).

he method we now present is rapid, le, sufficiently accurate, and thus suitable as a clinical routine method. From 1 ml of serum the calcium value is obtained within about ½ hr. with an accuracy of 2 per cent. As it is necessary, especially in children's clinics, to practice micro-methods, we have made a modification of this method for micro amounts, the necessary serum quantity being 0.1 ml.

METHOD

Solutions.

All solutions are made in doubly distilled water unless otherwise stated.

3.5 per cent ammonium-oxalate solution.

1-n hydrochloric acid.

m/200 EDTA. 1.860 g pro anal. disodium salt of ethylene-diamine-tetraacetic acid ad 1000 ml.

m/200 magnesium solution. 1.020 g pro anal. $MgCL_2 \cdot 6\,H_2O$ ad 1000 ml.

Buffer solution:

70 ml conc. ammonium (25 per cent NH_3) ad 1000 ml. It is kept in a well stoppered flask.

Indicator:

A. 100 mg of eriochromeschwarz T plus 900 mg hydroxylamine-hydrochloride ($NH_2OH \cdot HCl$) are dissolved in 20 ml of methyl alcohol. Instead of hydroxylamine conc. ammonium can be used.

B. 0.05 per cent methyl red in ethanol.

Indicator mixture: 1 part A plus 2 parts B.

Standard solution: 1 g of dried pro anal. $CaCO_3$ is dissolved in 25 ml of pro anal. conc. HCl and diluted to 1000 ml with doubly distilled water. 1 ml of this solution contains 0.4 mg of calcium.

Apparatus.

Centrifuge tubes (of at least 10 ml) and a good centrifuge.

Graduated pipettes and 1 ml ball pipettes. When measuring the serum and the EDTA solution, only calibrated 1 ml ball pipettes should be used.

1 ml micro burette.

Control of the solutions

A few ml of aq. redest., 1 ml of 1-n hydrochloric acid, exactly 1 ml of EDTA solution, 5 ml of buffer and 1—2 drops of the indicator solution are transferred to a small Erlenmeyer flask. The solution is now of a beautifully green color. The mixture is titrated with the magnesium solution to greyish red, an additional drop gives a clear red color. An excess of the indicator makes the shift point more difficult to observe.

Standard curve

The result of the determination can be calculated according to the method presented by Nielsen (1952), but in continued work the use of factors and calculation schemes is most inconvenient. We have made a standard curve based on known calcium values, the ordinate indicating the amount of ml magnesilm solution needed for the titration and the abscissa indicating the corresponding mg per cent Ca. This has been a very convenient and rapid solution of the problem.

In order to calculate the standard curve, the calcium standard solution is diluted 1:20 (= 2 mg Ca/100 ml) in doubly distilled water. 1 ml of 1-n HCl, the desired amount of the diluted calcium solution — 1—10 ml corresponding to 2—20 mg per cent calcium — exactly 1 ml of EDTA solution, 5 ml buffer and 1—2 drops of indicator are transferred into a small Erlenmeyer flask. The mixture is titrated with magnesium solution to a greyish red color.

When 1 ml of EDTA solution is used, at most 20 mg per cent Ca can be determined. This amount is enough in most cases, but if so desired 2 ml of EDTA solution can be used, thus corresponding to 40 mg per cent Ca.

Procedure

1 ml of aq. redest., exactly 1 ml of the test serum, and 1 ml of ammonium oxalate are measured in a centrifuge tube. The tube is shaken with care until a cloudy precipitate is formed, whereafter it is left for one or two minutes. A longer standstill does no harm. The precipitate is centrifuged for 10—15 min. The clear fluid is carefully poured away. The white precipitate on the bottom of the centrifuge tube is dissolved in 1 ml of 1-n hydrochloric acid. If the tube is slightly shaken the dissolving is very easy. (Now the content of the centrifuge tube is rinsed into a small Erlenmeyer flask with 2×2 ml of aq. redest.) Exactly 1 ml of EDTA solution, 5 ml of buffer and 1—2 drops of the indicator are added. The mixture is titrated in the same way as above according to the directions for the standard curve to a greyish red color.

If the titration is performed in the centrifuge tube, which is recommended, the parenthethis should be omitted.

It the test serum is suspected to contain a considerable amount of calcium, *i. e.* more than 20 mg per cent, 2 ml of EDTA-solution is used.

The answers are read from the standard curve.

Precipitation of calcium from serum as oxalate.

t is mentioned in the previous methods when calcium is precipitated from serum solution should, after the ammonium late has been added, stand for at least hour. We made a series of experiments which we, after the addition of the ammo- n oxalate, left the centrifuge tubes stand- at room temperature for from 1 min. to hours. In these experiments we used ed serums from several patients.

ble I. *Influence of time in precipitation calcium from serum with ammonium late. In all other respects the determina- ions are performed according to the above method.*

Time	mg per cent Ca
1 min.	7.9
5 »	8.0
10 »	8.0
15 »	7.9
30 »	8.0
60 »	8.0
17 hours	8.0

The accuracy of the method

rom serum from a patient 10 calcium erminations were performed simultane- ly according to the method presented ve. The following results were obtained:

1) 9.9 mg % Ca
2) 9.8 »
3) 9.9 »
4) 9.8 »
5) 9.9 »
6) 9.9 mg % Ca
7) 9.7 »
8) 9.8 »
9) 9.6 »
10) 9.9 »

We calculated the average error from the highest (1) and the lowest (9) values of the series according to the following formula:

$$\sigma = \sqrt{\frac{\varepsilon (M - x)^2}{n - 1}}$$

in which M = arithmetical average, n = number of experiments (in this case 2) and x = the value of one experiment. Thus the average error is 0.21 mg per cent Ca.

The probable error is

$$\varepsilon = 0.675 \cdot \frac{\sigma}{\sqrt{n}}$$

i. e. 0.10 mg per cent Ca.

Further experiments

In the present method the surplus EDTA is titrated back. We also tried to titrate directly with EDTA solution. The color shift is not, however, so easy to observe as in the first experiment.

We also tried to perform the determination directly from the serum without precipitating the calcium. In this procedure the determination of calcium (Ca + Mg) ought to be more rapid still, but it is difficult to notice the end point.

Comparison with old methods

The modified method of Kramer-Tisdall (Clark & Collip 1925) is apparently the most common method for determination of calcium and we, therefore, carried out comparative determinations according to this method and ours.

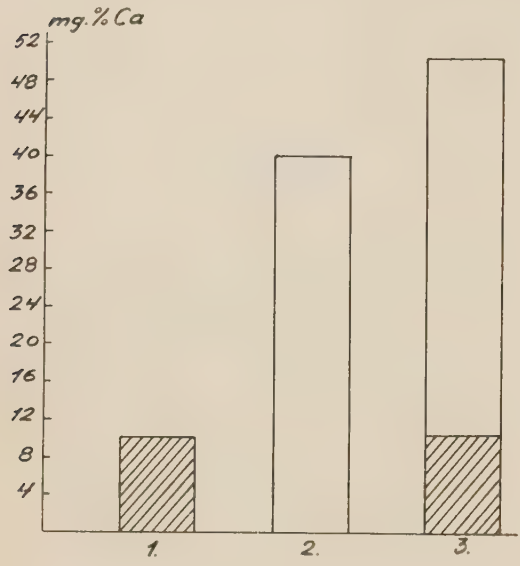

Fig. 1.

1. Determination from serum; result 10.1 mg per cent Ca.
2. Determination from pure calcium solution containing 40 mg per cent Ca. 40.2 mg per cent Ca was obtained.
3. Item 1, plus item 2. 50.1 mg per cent Ca.

Table II. *Parallel calcium determinations from the same serum samples. Comparison between the Kramer-Tisdall* (Clarck & Collip 1925) *and the EDTA method presented above.*

Kramer-Tisdall	EDTA	Kramer-Tisdall	EDTA
10.6	10.5	14.8	14.8
9.8	9.4	11.0	10.7
10.5	10.5	11.0	10.9
10.0	9.7	11.8	11.8
10.3	10.3	11.1	11.1
10.2	9.8	15.3	15.3
9.7	9.7		

Influence of hemolysis

Hemolyzed sera are often brought to the laboratories, and it is, of course, very important to know whether it is worth while

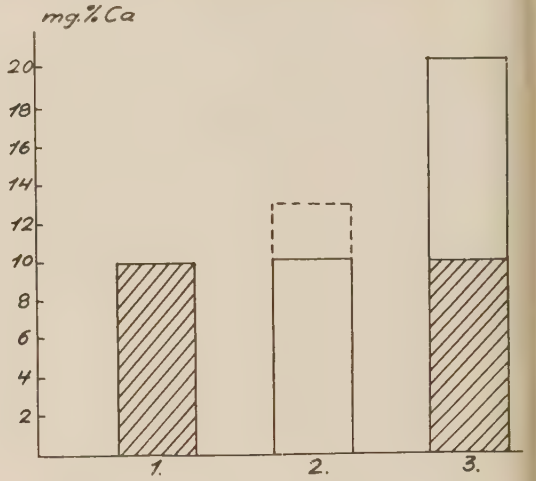

Fig. 2. Influence of magnesium in serum on the determinations.

1. Determination from serum; result 10.0 mg per cent Ca.
2. Determination from pure solution (10.0 mg per cent Ca + 2.9 mg per cent Mg). Result 10.2 mg per cent; without precipitating with oxalate 12.8 mg per cent.
3. Item 1, plus item 2. 20.2 mg per cent.

to perform a determination from these samples.

We prepared hemolyzed sera for our experiments as follows: We added a few drops of ether to the blood sample immediately after it had been drawn, shook the mixture violently, and the cells were centrifuged down at once. The serum obtained was dark red. From this serum a haemoglobin determination was made and the result was 0.95 g per cent of haemoglobin. As the patient's Hgb was 12 g per cent this means that 8 per cent of the red blood cells had disappeared. When the calcium determinations were carried out from this serum and the normal serum of the same patient, the result was as follows:

mal serum	To 8 per cent hemolyzed serum
10.25	9.85 mg per cent Ca

he only slightly lower value of the hemo-d serum depends apparently on the fact when the red blood cells disappear the me of the serum increases.

s the difference between this considerably olyzed serum and the normal serum was nsignificant, it can be said that hemolysis s not influence the determination of cal-n when this method is employed.

Micro modification

tions:

he same solutions as those in the macro hod presented above are used.

aratus:

all 3—4 ml centrifuge tubes (small test ubes can also be used).

ml pipettes for measuring the serum, and .5 ml and 1 ml graduated pipettes.

teur pipettes with the point drawn to a apillary.

ker. The mixing was performed so that uring the titration — with the point of ne byrette in the solution — small air ubbles were transmitted through a capil-ary.

ml micro byrette, preferably two samples, ne for adding EDTA the other one for tration with magnesium solution.

Procedure

.2 ml of aq. redest., exactly 0.1 ml of um and 0.2 ml of ammonium oxalate are sured in the centrifuge tube, mixed well centrifuged. The clear fluid is poured y and the small amount of precipitate on the bottom of the centrifuge test tube is dissolved in 0.2 ml of 1-n hydrochloric acid. 1 ml of buffer and exactly 0.1 ml of EDTA solution added, the best way of doing it is by using a micro burette.

The solutions are mixed and a micro drop of indicator is added. The mixture is titrated from the micro-burette with magnesium solution until the color changes from green to a reddish color. The results are read from a standard curve exactly as in the macro method.

Having made several determinations from the same serum sample by employing the above macro method as well as the micro method, the error for the micro modification is calculated to about 5 per cent in comparison with the macro method.

We have made solution determinations according to the above method also from urine, cerebrospinal and puncture fluids as well as from serum.

SUMMARY

The method for the determination of calcium in serum presented above is based on the employment of ethylene-diamine-tetra-acetic acid. By means of a suitable buffer and indicator the method is made rapid and the end point of the titration clearly visible. The result is obtained within ½ hour from the moment the serum is brought to the laboratory. In routine work the error is about 2 per cent when the determination is made from 1 ml of serum. When the determination is made from 0.1 ml of serum the error is about 5 per cent. The determination is not noticeably influenced by even considerably hemolyzed sera.

By means of the above method calcium can be determined also in other biological fluids than serum.

The method has been used as clinical routine method at our clinic for more than 6 months.

ACKNOWLEDGMENT

I wish to express my very best thanks to Dr. Greta Hammarsten, chief physician of the laboratories of the Södersjukhuset in Stockholm, for her kind supervision of my first basic experiments in 1953. I also wish to thank Dr. Ivar Halldorff, laboratory physician at the same clinic, for his valuable advice.

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PENICILLIN CLEARANCE AS KIDNEY FUNCTION TEST, DETERMINATIONS WITH AND WITHOUT COLLECTION OF URINE[1, 2, 3]

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(Received for publication July 5, 1954)

patients with ureterocolic anastomoses
letermination of renal clearances cannot
nsed upon direct measurements of the
nt of clearance substance excreted dur-
n definite period since a quantitative
ery of such substances from the colon
t feasible. For the study of such cases
ave therefore adopted a method that
not necessitate collection of the urine.

constant infusion method for clearance determination

e principle, suggested by Earle & Ber-
(1946) is based upon the following
nption: If a substance that is excreted
in the urine and not converted or de-
ed in the organism is injected at a con-
rate, a constant plasma level must be
lished when the distribution has become
lete and the amount of the substance
ted per time unit will then be equal to
mount excreted through the kidneys per
unit. The clearance can consequently be calculated from the formula $C = IV/P$ where IV is the amount injected per minute and P is the plasma concentration.

Determinations of the inulin clearance according to this principle have been published (Pers 1953), but it seemed desirable to be able to obtain additional information on the functional state of the tubular cells.

Tm-determinations for a secretion substance with the constant infusion method (Berger, Farber & Earle, 1948) were, however, abandoned, partly because an advance knowledge of the secretory capacity and the filtration rate is required in each case in order to calculate the correct dosage of the test substance, and partly because the very large amounts which would have to be supplied during the interval needed to establish equilibrium would involve a risk and be prohibitive also for economical reasons.

It was felt, however, that information of similar value could be provided by determination of the maximum clearance of a secretion substance, since it has been shown by Raaschou (1948) that the maximum clearance and Tm for diodrast are proportionally reduced in pyelonephritis, which probably is the main factor responsible for renal impairment in cases with ureterocolic anastomoses.

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y, Stockholm, May 26, 1954.

Reason why penicillin has been chosen as test substance

There is much evidence that penicillin is excreted by the renal tubles by a similar secretory mechanism as p-aminohippuric acid (PAH) and diodrast. Penicillin has maximum clearance values of the same order of magnitude as these substances and its excretion is depressed on simultaneous administration of either of them (Rammelkamp & Bradley 1943, Beyer, Woodwad, Peters, Verwey & Mattis 1944).

In the case of diodrast and PAH the ratio between the self-depression limit and the lowest measurable plasma concentration is only about 10 : 1. For this reason the plasma concentration cannot be kept within the useful range by means of the constant infusion method with fixed dosage (Pers 1953), when measurements are to be performed on patients with widely differing clearance values.

Penicillin, however, seemed to offer distinct advantages in this respect, since it has been reported by Eagle & Newman (1947) that in two rabbits the self-depression limit was observed between 160 and 1600 units/ml or about 1000 times the lowest measurable plasma concentration.

A necessary prerequisite for applying the constant infusion method to penicillin clearance determinations ist hat the substance is exclusively excreted by the kidneys. The demonstration (Anderson & Brodersen 1949) that considerable amounts of penicillin are excreted in the bile might therefore appear to exclude the application of penicillin to this method. The fact that penicillin is easily absorbed from the duodenum (Rammelkamp & Keefer 1943, McDermott, Bunn, Benoit, Du Bois & Reynolds 1946) makes it likely, however, that the penicillin excreted in the bile is not definitly lost but largely returned to the body.

This assumption is in agreement with an observation of Beyer (1944) who found 98 per cent recovery of penicillin in the urine from dogs, but it should be mentioned that less complete recoveries have been reported by other investigators (Rammelkamp & Keefer 1943, Jensen, Møller & Overgaard 1945).

Table I.

Author	Penicillin	Number of determinations	Subjects	Clearance ml/min
Rantz & Kirby 1944	Na-Penicillin	7	healthy adults	755—1120
Jensen et al. 1945	Na-G-Penicillin	1	» »	580
Eagle & Newman 1947	Na-G-Penicillin	5	» »	560
Barnett et al. 1948	G-Penicillin	14	children & infants	596(per1.73sq.m
Bryner et al. 1949	Potassium-Penicillin in wax and oil	11	5 healthy adults	623
» » »	—»—	11	patients with renal disease	136-423
» » »	—»—	15	patients without renal disease	220-1214

revious investigations on the clearance of penicillin

he maximum plasma clearance of peni- n in human subjects has been measured everal previous studies. The results are n in Table I.

ome authors suggest the use of penicillin rance as a clinical kidney function test, so far it does not seem to have received tical application. This is probably due to facts that when collection of urine is ible, penicillin clearance does not furnish e information than obtainable with the rances of phenol red, diodrast or PAH; the determination of penicillin requires ast some practice in bacteriological tech- e, and that the accuracy of the method ied for its determination in most of the ies cited (Rantz' dilution method) does always seem to be entirely satisfactory.

PERSONAL INVESTIGATIONS

Method used for the determination of penicillin

A. Jensen's modification (1945) of nming's agar-cup method was employed determination of penicillin in urine and ma.

he plasma concentrations practically ys exceeded 1 unit/ml. In such cases the rminations were performed on plasma ted 1:4 and no correction for self- bition by plasma was necessary. In a few s lower plasma concentrations (down to unit/ml) were determined and undiluted ma was then used and a correction for inhibition had to be applied.

n more than 300 determinations carried in triplicate the standard deviation from mean was ± 6.0 per cent.

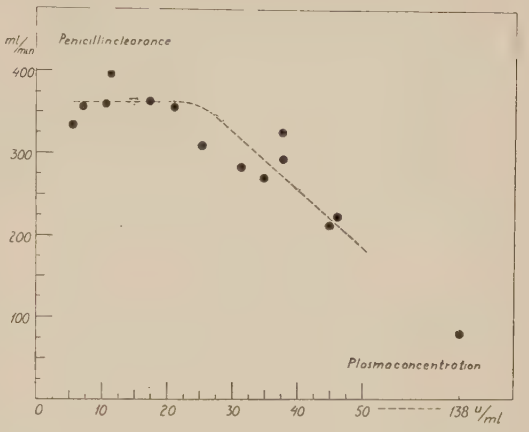


Fig. 1. Determination of the self-depression limit for Na-G-penicillin.

Estimation of the self-depression limit for Na-G-penicillin

As mentioned above, Eagle & Newman (1947) found the self-depression limit for Na-G-penicillin in rabbits to be somewhere between 160 and 1600 units/ml.

Since no data for human subjects were available in the literature, a determination of the self depression limit was made in a healthy adult subject. Na-G-penicillin was administered by constant intravenous infusion which was step-wise reduced (from 28,000 to 2,200 units/min.) over a total period of 8 hours. Within this period the clearance was determined in 14 intervals, each of 15 minutes duration. Blood samples were taken at the beginning and the end of each interval.

Maximum clearance values were obtained at plasma concentrations up to 25 units/ml (Fig. 1). At higher concentrations the clearance gradually declined, and at the highest plasma level achieved (138 units/ml) it became of the same order of magnitude as the glomerular filtration rate.

Although this suggests that the self-depression limit for the clearance of Na-G-penicillin in man is considerably lower that the values observed by Eagle & Newman (1947) in rabbits, it still leaves a wide range of usable plasma concentrations (0.25 to 25 units/ml) for maximum clearance determinations.

Dosage of penicillin employed for clearance determinations by the constant infusion method

The penicillin dosage required to maintain plasma levels at suitable magnitudes with constant intravenous infusion was determined by the same approach as used for determining the dosage of inulin (Pers 1953).

The following dosage was found suitable: A priming dose of 600,000 units was injected over 20 minutes, and 70 minutes later a sustaining infusion of 1000 units/min was commenced and maintained for about 5 hours with hourly control of the plasma concentration, the constancy of which indicated that complete equilibrium was present.

With this dosage and at clearances between 75 and 1100 ml/min, plasma levels ranging from 15 to 1 unit/ml were established, *i. e.* a suitable range for maximum clearance determinations.

Equilibrium plasma concentrations will not be reached with this dosage within the experimental time chosen, if the clearances are lower than about 75 ml/min which therefore must be taken as the lower limit for reliable measurements of penicillin clearance by infusion in the present study.

Series of clearance determinations

The clearance of Na-G-penicillin has been determined in 42 patients with normal or reduced kidney function.

In 5 cases clearance was determined with collection of urine at rapidly decreasing plasma concentration following a single intravenous injection. The plasma concentration in the middle of the period was used in the calculation (UV/P).

In order to control the applicability of the constant infusion technique for determinations of penicillin clearance, the latter was calculated in 14 cases, both from the rate of infusion and from the rate of urinary excretion under maintenance of the constant plasma levels.

The remaining 23 cases were patients who had ureterocolic anastomoses and in whom, consequently, clearances were determined only by the constant infusion method.

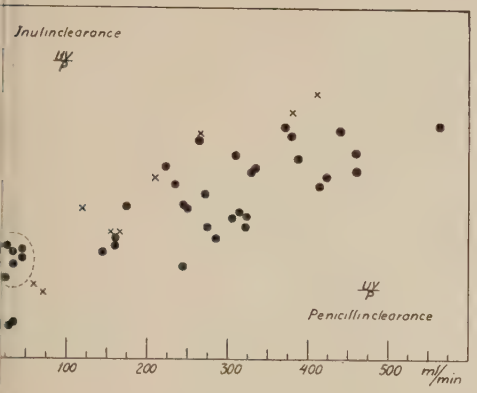
Simultaneous determinations of inulin clearance were made in all the above mentioned cases by corresponding technique and the relation between the impairment of the two clearances will be discussed below.

RESULTS AND DISCUSSION

Determinations based on collection of urine

The penicillin clearances measured by collection of urine in 3 successive periods showed a standard deviation of $\pm$ 10.5 per cent from the mean.

In Figure 2 are shown the results of all the determinations of penicillin and inulin clearance based upon collection of urine. It is apparent that in those experiments (indicated by cross-signs) where penicillin clearance was measured at rapidly decreasing



. 2. Penicillin clearance determined with collec- of urine, compared with simultaneous inulin rance. Cross-signs indicate measurements at ng plasma concentration. Dots indicate measurements at constant plasma level.

sma concentrations, the penicillin clear- e values observed are lower in proportion he insulin clearance than is the case when h clearances were measured at constant sma levels (indicated by dots).

t is most likely that in the first group the icillin clearance values were actually her than measured, since calculations re based upon venous plasma concentra- ns. As Brun, Hilden & Raaschou (1949) e emphasized for diodrast, venous con- trations at falling plasma concentrations l become significantly higher than the rial concentrations, resulting from a con- uous entrance of the substance into the od during its passage through extra-renal ues.

Among the patients were 2 cases with remely reduced tubular function, one of ch has recently been published as a typ- case of Fanconi's syndrome, (Dragsted Hjort 1953). In these (encircled by a ted line in Fig. 2) the penicillin clearance

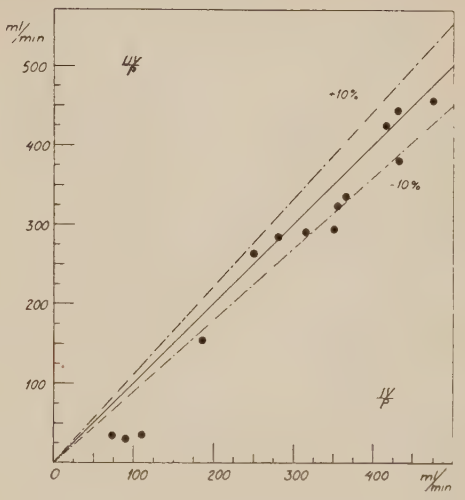


Fig. 3. Comparison between the penicillin clearance determined with constant infusion (IV/P) and with collection of urine (UV/P).

was found to be considerably lower than the inulin clearance. Assuming the measurements to be correct, the most likely explanation seems to be that tubular secretion of penicillin was almost completely abolished. If so, the penicillin clearance would be expected to be about half the inulin clearance value, since only some 50 per cent of the penicillin in plasma is filtrable (Tompsett, Schultz & McDermott 1947).

Determinations based on constant infusion

It is apparent from Fig. 3 that, at clearances from about 100 ml/min and upwards, conformity was observed between penicillin clearance values calculated from the rate of infusion (IV/P) and those calculated from the rate of urinary excretion (UV/P).

This indicates that penicillin is almost exclusively eliminated by renal excretion in these cases. At very low UV/P values (below

75 ml/min) the conformity does not hold, as the IV/P values are measured too high. As mentioned above, one possible explanation for this discrepancy is that equilibrium had not been reached. Secondly it is probable that bilary excretion achieves importance in these cases as it becomes responsible for a greater fraction of the total elimination.

The last diagram (Fig. 4) shows all the clearances measured by the constant infusion method. Those obtained in patients who passed the urine in the normal way are indicated by black squares and those from patients with ureterocolic anastomoses by open squares.

The fact that the penicillin/inulin clearance ratio was not found to be lower in the latter of these groups than in the first, is taken as evidence that no appreciable return of penicillin to the body occurs from the colon. The correctness of this was further supported by the fact that in a separate experiment, where 600,000 units of penicillin were introduced into the colon, no penicillin could be detected in the plasma within 6 hours.

The signification of penicillin clearance

No special investigation has been made here to determine the normal value for the clearance of Na-G-penicillin, but in several of the patients studied there was no evidence of renal disease. From the average penicillin /inulin clearance ration of 4:1 obtained at penicillin clearances above 200 ml/min (and indicated in Fig. 4 by the dotted line), the normal value corresponding to an inulin clearance of 120 ml/min may be estimated to be about 480 ml/min.

Taking 600 ml/min as the corresponding figure for maximum diodrast clearance and

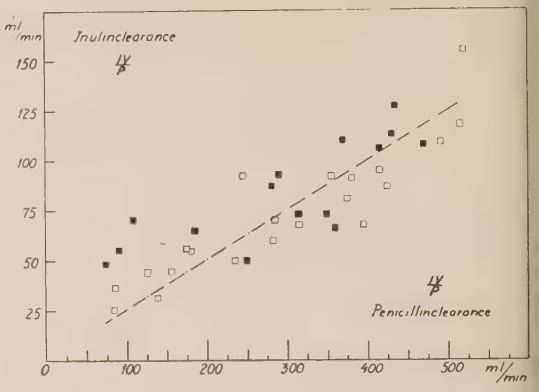


Fig. 4. Penicillin clearance determined without collection of urine, compared with simultaneous inulin clearance. The open squares indicate measurements in patients with ureterocolic anastomoses.

0.80 as the normal extraction ratio of diodrast, the penicillin extraction ratio would be about 0.65. As mentioned above, the protein-bound fraction of penicillin in plasma is about 50 per cent. In Table II these values are compared with those reported for other substances excreted by tubular secretion.

Table II.

	Clearance	Extraction ratio	Unbound fraction in plasma
Phenol red	380	0.55	20 %
Penicillin	480	0.65	50 »
Diodrast	600	0.80	75 »
PAH	600	0.90	80 »

It appears that the clearances and the extraction ratios are correlated to the unbound fraction. This correlation may intelligibly be explained by assuming that the substances which possess the smallest unbound fractions are also those in which the detachment of the substance from the plasma proteins

urs at the lowest rate during the passage blood through the kidney.

t is, however, also possible that differes in the rates of diffusion through the itubular spaces are of some importance. t is evident that, due to the low extraction o, maximum penicillin clearance does not ord a measure of the renal plasma flow, n in healthy subjects. On the other hand, eems reasonable to believe that the maxim clearance of PAH or diodrast will be ject to similar relative changes under hological conditions as that of penicillin. erefore relative reductions in penicillin rance should provide the same informa- as is obtained from relative reductions PAH or diodrast clearances.

As is well known, subnormality of PAH diodrast clearance values may be due ier to lowering of the renal plasma flow to impairment of the secretary ability of tubular cells, with consequent reduction the extraction ratio. Obviously the same factors may lead to reduction in peni- in clearance, but we are unable to decide what extent each of them were contribut- to the reductions observed in the present dy.

CONCLUSIONS AND SUMMARY

In 42 patients with normal or impaired kidney function the clearances of Na-G-penicillin and inulin were determined simultaneously.

The self-depression limit for Na-G-penicillin was found to be about 25 units/ml in a healthy adult subject. This permits determination of the maximum clearance at plasma concentrations up to about 100 times the smallest accurately measurable concentration.

3. Penicillin was therefore—in contrast to PAH or diodrast — suitable for the constant infusion method with a fixed dosage by which clearance determinations without collection of urine can be performed, *e. g.* in patients with ureterocolic anastomoses.
4. By employment of the constant infusion method with a fixed dosage of Na-G-penicillin, clearances between 75 and 1100 ml/min could be measured with corresponding plasma levels ranging from 15 to 1 unit/ml.
5. When the clearance of penicillin was measured with collection of urine at falling plasma concentration, the values determined were higher than those found at constant plasma levels. The standard deviation for the penicillin clearance values in three successive periods was $\pm$ 10.54 per cent of the mean.
6. When the calculation of the clearance could be based upon the rate of infusion as well as upon the rate of urinary excretion, conformity was found for values higher than about 100 ml/min.
7. Penicillin clearance was found to be lower than inulin clearance in two cases with extremely reduced tubular function.
8. In cases with penicillin clearances above 200 ml/min, the average penicillin/inulin clearance ratio was 4 : 1. From this the normal value of Na-G-penicillin clearance was estimated to be about 480 ml/min.

9. The rather high protein binding of Na-G-penicillin in plasma may possibly explain that the maximum penicillin clearance is lower than those of PAH and diodrast.
10. It is assumed that reduction in PAH or diodrast clearances will be paralleled by similar relative reductions in penicillin clearance.

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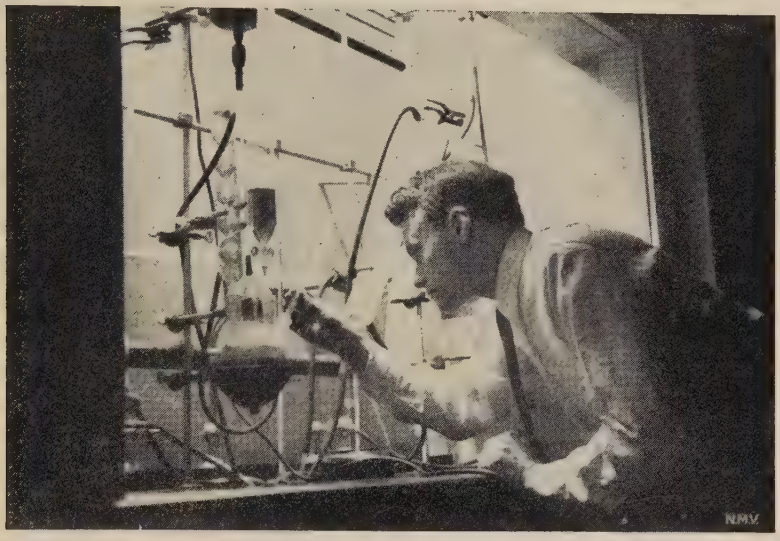

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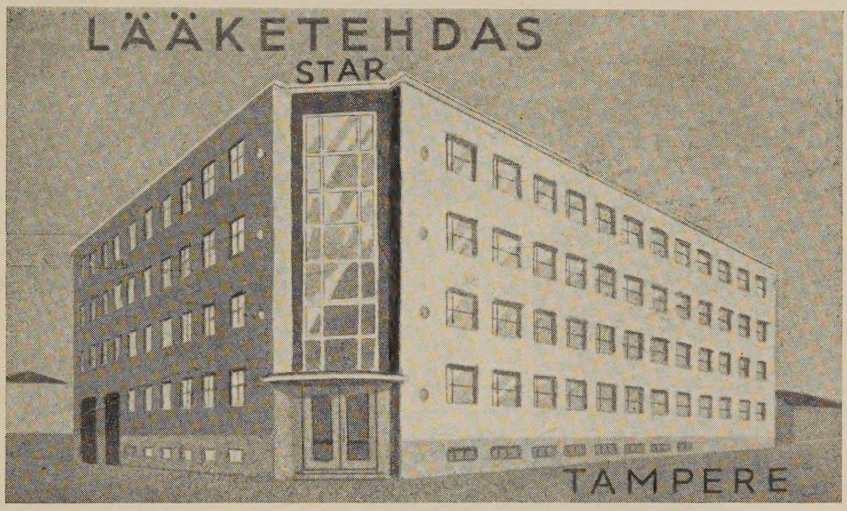

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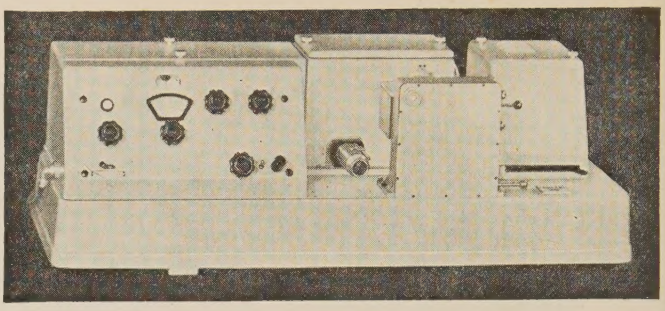

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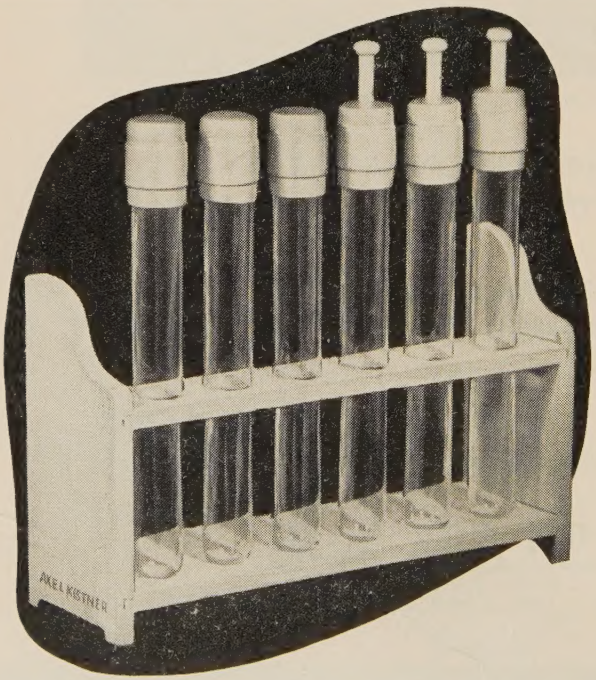

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